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EXHIBIT A



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(21) International Application Number: PCT/IB97/01440 (22) International Filing Date: 30 October 1997 (30.10.97) (30) Priority Data: 9622660.0 31 October 1996 (31.10.96) GB (71) Applicant (for all designated States except US): CHIRON S.P.A. [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT). (72) Inventors; and (75) Inventors/Applicants (for US only): PIZZA, Mariagrazia [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT). GIULIANI, Marzia, Monica [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT). RAPPUOLI, Rino [IT/IT]; Via Fiorentina, 1, I-53100 Siena (IT). (74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: IMMUNOGENIC DETOXIFIED MUTANT E. COLI LT-A TOXIN (57) Abstract <p>An immunogenic detoxified protein is provided which comprises the amino acid sequence of subunit A of an <i>E. coli</i> heat labile toxin (LT-A) or a fragment thereof in which at least amino acid Ala-72 of the A subunit is mutated, preferably by substitution with Arg. The toxoid is useful as vaccine against an enterotoxigenic strain of <i>E. coli</i> and is produced by recombinant DNA means by site-directed mutagenesis. It is also an effective adjuvant.</p>		

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IMMUNOGENIC DETOXIFIED MUTANT E. COLI LT-A TOXIN

Field of the invention

5 The present invention relates to immunogenic detoxified heat labile toxin proteins (LT) produced by enterotoxigenic strains of *Escherichia coli* (*E.coli*) wherein at least amino acid Ala-72 of the A subunit is mutated, and to their use in vaccines which are useful in the prevention or treatment of
10 enterotoxigenic *E.coli* infections and as adjuvants for other immunogenic proteins. Toxoids of the invention can be suitably produced using recombinant DNA techniques by site-directed mutagenesis of DNA encoding the wild-type toxins.

15

Background to the Invention

Heat-labile enterotoxin (LT), produced by enterotoxigenic strains of *E.coli*, and cholera toxin (CT), produced by
20 *V.cholerae* strains, are the causative agents of traveller's diarrhoea and cholera, respectively [Spangler (1992) *Microbiol Rev* 56:622]. LT and CT show 80% homology in the primary structure and an identical tertiary structure. They are composed of two functionally distinct domains: the
25 enzymatically-active A subunit and the B pentamer, which contains the receptor-binding site. The A subunit ADP-ribosylates the target protein Gs, a GTP-binding protein which regulates the intracellular levels of cAMP [Rappuoli & Pizza (1991), in *Sourcebook of bacterial protein toxins*,
30 Academic Press NY]. Enhancement in cAMP levels can alter ion transport, inducing secretion of water and chloride ions into the intestine.

CT and LT are both powerful immunogens and potent mucosal
35 adjuvants when co-administered with antigens at the mucosal level [eg. Jackson et al. (1993) *Infect Immun* 61:4272; WO95/17211]. Immunogenicity and adjuvanticity of wild-type CT and LT have been extensively studied in animals [eg.

Rollwagen et al. (1993) *Vaccine* 11:1316], but their toxicity has precluded their use in humans. In an attempt to overcome the problems generated by the use of active holotoxins, two different approaches have been followed, one based on the
5 use of the B pentamer, the non-toxic domain of the holotoxin [eg. Holmgren et al. (1992) *Vaccine* 10:911], and the other based on the generation of genetically detoxified derivatives of LT and CT [eg. Fontana et al. (1995) *Infect Immun* 63:2356]. Site-directed mutagenesis on both A and B
10 subunits has provided a tool to explore the basis of the immunological and adjuvant responses induced by these molecules.

Examples of such experiments can be found in:

- 15 - Harford et al. [*Eur J Biochem* (1989) 183:311] made LT-A carrying Ser-61-Phe and Gly-79-Lys substitutions.
- Tsuji et al. [*J Biol Chem* (1990) 265:22520] produced LT-A carrying a Glu-112-Lys substitution.
- Burnette et al. [*Infect Immun* (1991) 59:4266] produced
20 CT-A carrying a Arg-7-Lys substitution. This work can also be seen in WO92/19265.
- WO93/13202 described non-toxic CT and LT carrying mutations at Val-53, Ser-63, Val-97, Tyr-104, and Pro-106

25 A mutant in the receptor binding site of the B subunit of LT, the G33D mutant, has been reported to lack the immunological properties of the native B subunit [Nashar et al. (1996) *PNAS* 93:226], suggesting that binding to the receptor is important for the immunogenicity. It has also
30 been shown that non-toxic derivatives of LT carrying mutant A subunits retain the immunological properties of wild-type LT [eg. Magagnoli et al. (1996) 64:5434], suggesting that ADP-ribosylation activity is not essential for immunogenicity.

35

In relation to adjuvanticity, much data has been generated but many questions remain unanswered. Some studies have suggested that LT-B and CT-B have adjuvant activity, but the

conclusions drawn have been compromised by contamination with active toxin [Wilson et al. (1993) *Vaccine* 11:113]. Studies with recombinant LT-B and CT-B have suggested that they behave as poor mucosal adjuvants [eg. Douce et al. 5 (1997) *Infect. Immun.* 65:2821]

Attempts to define the role of ADP-ribosylation activity in the adjuvanticity of LT has generated conflicting results. Lycke et al. [*Eur J Immunol* (1992) 22:2277] have described 10 a non-toxic LT derivative (LT-E112K) which, when administered with KLH by oral route in mice, lacked the adjuvant properties of wild-type LT, thus suggesting that adjuvant activity is linked to ADP-ribosylation activity. LT derivatives such as LT-K7 and LT-K63 [eg. Douce et al. 15 (1997) *supra*; Douce et al. (1996) *PNAS* 92:1644; DiTommaso et al. (1996) *Infect Immun* 64:974], however, which are devoid of toxicity both *in vitro* and *in vivo*, have been shown to be able to elicit an antibody response against a co-administered antigen in intranasally immunised mice. 20 LT-K63 has been shown to induce measles-specific CTL response after intranasal immunisation with a synthetic peptide [Partidos et al. (1996) *Immunol* 89:483], and strongly enhances protection against *H.pylori* following intragastric immunisation with *H.pylori* antigens. The antibody titres 25 induced by these non-toxic LT mutants were lower than those obtained with wild-type toxin, however, and were only detected after two mucosal immunisations [Douce et al. (1997) *supra*].

30 It is an object of the invention to provide forms of LT which are detoxified, so that they might be suitable for use in humans, but which retain the adjuvant and immunogenic properties of LT as far as possible.

35 Summary of the invention

According to a first aspect of the present invention, there is provided an immunogenic detoxified protein comprising the

amino acid sequence of subunit A of an *E.coli* heat labile toxin (LT-A), or a fragment thereof, wherein at least amino acid Ala-72 in said sequence or fragment is mutated.

- 5 As used herein, the term "detoxified" means that the toxoid exhibits a reduction in toxicity relative to the wild-type toxin. The toxicity may be measured in mouse cells, CHO cells, by evaluation of the morphological changes induced in Y1 cells, or preferably by the rabbit ileal loop assay. As
10 measured in Y1 cells, for instance, "detoxified" means that the toxoid exhibits a reduction in toxicity relative to the wild-type toxin of greater than 10,000-fold.

Any residual toxicity should be sufficiently low for the
15 protein to be used in an effective immunogenic composition without causing significant side effects. Certain mutants within the scope of the invention may possess zero toxic activity.

- 20 As used herein, the term "residual toxicity" means that the detoxified immunogenic protein may retain a measurable toxicity. More particularly the level of toxicity may be optimised in a benefit/side-effect trade-off to maximise immunogenicity and/or adjuvanticity whilst maintaining a
25 sufficiently low toxicity to be tolerated by the subject after administration.

Thus, although these proteins are detoxified in the sense of having a much lower toxicity than the wild-type protein,
30 traces of the enzymatic activity responsible for toxicity may remain. The mutation causes a decrease in toxicity of which makes the toxoid suitable for human use.

Most preferably the toxicity of the toxoid is reduced
35 relative to its natural occurring counterpart by greater than 10,000 fold as measured by the evaluation of the morphological changes induced in Y1 cells or greater than 10 fold as measured by the rabbit ileal loop assay, performed

as described herein.

The term "toxoid" as used herein means a detoxified mutated toxin protein.

5

In this specification, references to LT encompass the various naturally occurring strain variants as well other variants encompassing mutations which do not significantly alter the properties of the subunits or of the assembled
10 holotoxin. These mutations may be, for instance, conservative amino acid changes which do not affect the assembly of the holotoxin.

References to LT-A also encompass fragments of LT-A provided
15 that the fragment contains Ala-72.

Most importantly, the toxoid must remain immunologically cross-reactive with the toxin from which it is derived.

20 The immunogenic protein may be LT subunit A toxoid, but is preferably an assembled holotoxoid comprising a LT-A toxoid and five LT-B subunits, which may themselves be wild-type or mutated.

25 It will be appreciated that in derivatives of LT-A, such as fragments, or in LT-A proteins of different *E.coli* strains, the amino acid residue to be mutated is that which corresponds to Ala-72 as defined for LT-A in Domenighini et al. [*Molec. Microbiol.* (1995) 15:1165-1167]. Ala-72 is
30 located on the second turn of the alpha-helix in LT-A and faces the NAD binding site.

The mutation at Ala-72 may be a substitution, an insertion, or a deletion. Preferably it is a substitution with a
35 different amino acid. The most preferred mutation is the substitution of Ala-72 with arginine, for which the standard nomenclature is A72R.

Whilst the A72R mutant retains residual toxicity, other mutants within the scope of the invention may possess no toxicity, for example mutants with one or more mutations at other sites, or toxoids which are further detoxified by
5 chemical means.

The or each amino acid substituted for a wild type amino acid, whether at Ala-72 or elsewhere, may be a naturally occurring amino acid or may be a modified or synthetic amino
10 acid, provided that the mutant retains the necessary immunogenic and detoxified properties.

Substitutions which alter the amphotericity and/or hydrophilicity of the protein whilst retaining as far as
15 possible the steric effect of the substituted amino acid are generally preferred.

The toxoids of the invention may be synthesised chemically using conventional peptide synthesis techniques, but are
20 preferably produced by recombinant DNA means.

Preferably the toxoid is obtained in substantially pure form.

25 According to a second aspect of the invention, there is provided an immunogenic composition comprising an immunogenic detoxified protein of the first aspect of the invention and a pharmaceutically acceptable carrier. This immunogenic composition may be a vaccine against the
30 enterotoxigenic strains of *E.coli* itself and may thus optionally further comprise an adjuvant.

In addition to the properties typical of mutants devoid of enzymatic activity, the mutant proteins also exhibit
35 adjuvant activity.

Additionally, the immunogenic composition may further comprise a second antigen capable of raising an

immunological response to another disease. In such an alternative composition, the immunogenic detoxified protein of the invention can act as a mucosal adjuvant.

- 5 According to a third aspect of the invention, there is provided the use of a toxoid of the first aspect as an adjuvant.

According to a fourth aspect of the invention, there is
10 provided a method of vaccinating a mammal against an enterotoxigenic strain of *E.coli* comprising administering an immunologically effective amount of an immunogenic detoxified protein according to the first aspect of the invention. Optionally, the immunogenic detoxified protein of
15 the invention may act as an adjuvant for a second immunogenic protein administered separately, sequentially or with the immunogenic detoxified protein of the invention.

According to a fifth aspect of the invention, there is
20 provided a DNA sequence encoding an immunogenic detoxified protein according to the first aspect of the invention.

Preferably the DNA sequence encodes both the detoxified subunit A and subunit B in a polycistronic unit.
25 Alternatively, the DNA may encode only the detoxified subunit A.

According to a sixth aspect of the invention, there is provided a vector comprising a DNA sequence according to the
30 fifth aspect of the invention.

According to a seventh aspect of the invention, there is provided a host cell transformed with a vector according to the sixth aspect of the invention.

35

The host cell may be any host capable of expressing a DNA sequence according to the fifth aspect, but is preferably a bacterium, most preferably *E.coli*, suitably transfected to

produce the desired immunogenic detoxified protein.

In a further embodiment of the seventh aspect of the invention, the host cell may itself provide a protective
5 species, for example an *E.coli* strain mutated to a phenotype lacking wild type LT and expressing an immunogenic detoxified protein of the first aspect of the invention.

According to a eighth aspect of the invention, there is
10 provided a process for the production of an immunogenic detoxified protein according to the first aspect of the invention comprising culturing a host cell according to the seventh aspect of the invention.

15 According to a ninth aspect of the invention, there is provided a process for the production of DNA according to the fifth aspect of the invention comprising the steps of subjecting a DNA encoding a wild-type LT-A or a fragment thereof to site-directed mutagenesis.

20

According to a tenth aspect of the invention, there is provided a process for the formulation of an immunogenic composition according to the second aspect comprising
bringing an immunogenic detoxified protein according to the
25 first aspect of the invention into association with a pharmaceutically acceptable carrier, and optionally with an adjuvant.

According to a eleventh aspect of the invention, there is
30 provided a method for the prevention or treatment of disease in a subject, comprising administering to said subject an immunologically effective dose of an immunogenic composition according to the second aspect.

35 Industrial Applicability

The immunogenic detoxified protein of the invention may constitute the active component of a vaccine composition

useful for the prevention and treatment of infections by enterotoxigenic strains of *E.coli*. The immunogenic detoxified protein may also be used in a vaccine composition as a mucosal adjuvant. The compositions are thus applicable
5 for use in the pharmaceutical industry.

Description of the drawings

Figures 1 to 5 show the biochemical and biological
10 properties of wild-type LT and of mutants LT-A72R (also shown as "LTR72") and LTK63: Figure 1 is a chromatographic profile on a superdex column, with peak I corresponding to holotoxin and peak II to the EDTA in the buffer; Figure 2 is a Western blot of a trypsin digest experiment; Figure 3
15 shows the results of *in vitro* ADP-ribosylation of polyarginine; and Figures 4 and 5 show the results of *in vitro* and *in vivo* toxicity experiments, respectively.

Figure 6 shows serum anti-OVA antibody response after three
20 intranasal immunisations (indicated by arrows). Figure 7 shows the IgG subclasses after the third immunisation. Figure 8 shows the IgA levels after the third immunisation in serum samples (8A) and nasal washes (8B) [mean titre and standard deviation are shown].

25

Figure 9 shows serum anti-LT Ig (9A) and IgA (9B) responses.

Figure 10 shows OVA-driven proliferative responses. Background values (no OVA added to cultures) varied between
30 1000 and 3000 cpm.

Figure 11 shows the efficacy against systemic challenge of intranasal immunisation with wild-type LT and with mutants A72R and K63. Columns in 11B are mean titres and dots are
35 individual titres. Black dots show titres from mice immunised with LT_B and not protected against challenge.

Detailed Description of Embodiments of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See eg., Sambrook, et al., MOLECULAR CLONING; A LABORATORY MANUAL, SECOND EDITION (1989); DNA CLONING, VOLUMES I AND II (D.N Glover ed. 1985); OLIGONUCLEOTIDE SYNTHESIS (M.J. Gait ed, 1984); NUCLEIC ACID HYBRIDIZATION (B.D. Hames & S.J. Higgins eds. 1984); TRANSCRIPTION AND TRANSLATION (B.D. Hames & S.J. Higgins eds. 1984); ANIMAL CELL CULTURE (R.I. Freshney ed. 1986); IMMOBILIZED CELLS AND ENZYMES (IRL Press, 1986); B. Perbal, A PRACTICAL GUIDE TO MOLECULAR CLONING (1984); the series, METHODS IN ENZYMOLOGY (Academic Press, Inc.); GENE TRANSFER VECTORS FOR MAMMALIAN CELLS (J.H. Miller and M.P. Calos eds. 1987, Cold Spring Harbor Laboratory), Methods in Enzymology Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively), Mayer and Walker, eds. (1987), IMMUNOCHEMICAL METHODS IN CELL AND MOLECULAR BIOLOGY (Academic Press, London), Scopes, (1987), PROTEIN PURIFICATION: PRINCIPLES AND PRACTICE, Second Edition (Springer-Verlag, N.Y.), and HANDBOOK OF EXPERIMENTAL IMMUNOLOGY, VOLUMES I-IV (D.M. Weir and C. C. Blackwell eds 1986).

Standard abbreviations for nucleotides and amino acids are used in this specification. All publications, patents, and patent applications cited herein are incorporated by reference.

In particular, the following amino acid abbreviations are used:

Alanine	A	Ala	Arginine	R	Arg
Asparagine	N	Asn	Aspartic Acid	D	Asp
Cysteine	C	Cys	Glycine	G	Gly
Glutamic Acid	E	Glu	Glutamine	Q	Gln

	Histidine	H	His	Isoleucine	I	Ile
	Leucine	L	Leu	Lysine	K	Lys
	Methionine	M	Met	Phenylalanine	F	Phe
	Proline	P	Pro	Serine	S	Ser
5	Threonine	T	Thr	Tryptophan	W	Trp
	Tyrosine	Y	Tyr	Valine	V	Val

As mentioned above examples of the immunogenic detoxified protein that can be used in the present invention include
10 polypeptides with minor amino acid variations from the natural amino acid sequence of the protein other than at the sites of mutation specifically mentioned.

A significant advantage of producing the immunogenic
15 detoxified protein by recombinant DNA techniques rather than by isolating and purifying a protein from natural sources is that equivalent quantities of the protein can be produced by using less starting material than would be required for isolating the protein from a natural source. Producing the
20 protein by recombinant techniques also permits the protein to be isolated in the absence of some molecules normally present in cells. Indeed, protein compositions entirely free of any trace of human protein contaminants can readily be produced because the only human protein produced by the
25 recombinant non-human host is the recombinant protein at issue. Potential viral agents from natural sources and viral components pathogenic to humans are also avoided. Also, genetically detoxified toxin are less likely to revert to a toxic form than more traditional, chemically detoxified
30 toxins.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition.
35 Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, lipid aggregates (such as oil droplets or

liposomes) and inactive virus particles. Such carriers are well known to those of ordinary skill in the art. Additionally, these carriers may function as immunostimulating agents (adjuvants).

5

Preferred additional adjuvants to enhance effectiveness of immunogenic compositions include, but are not limited to: aluminum salts (alum) such as aluminium hydroxide, aluminium phosphate, aluminium sulfate etc, oil emulsion formulations, with or without other specific immunostimulating agents such as muramyl peptides or bacterial cell wall components, such as for example (1) MF59 (Published International patent application WO90/14837, containing 5% Squalene, 0.5% Tween® 80, 0.5% Span® 85 (optionally containing various amounts of MTP-PE (see below), although not required) formulated into submicron particles using a microfluidizer such as Model 110Y microfluidizer (Microfluidics, Newton, MA 02164), (2) SAF, containing 10% squalene, 0.4% Tween 80, 5% pluronic-blocked polymer L121, and thr-MDP (see below) either microfluidized into a submicron emulsion or vortexed to generate a larger particle size emulsion, and (3) RIBI™ adjuvant system (RAS) (Ribi Immunochem, Hamilton, MT) containing 2% Squalene, 0.2% Tween® 80 and one or more bacterial cell wall components from the group consisting of monophosphoryl lipid A (MPL), trehalose dimycolate (TDM), and cell wall skeleton (CWS) preferably MPL+CWS (Detox™), muramyl peptides such as N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (MTP-PE) etc., and cytokines, such as interleukins (IL-1, IL-2 etc) macrophage colony stimulating factor (M-CSF), tumour necrosis factor (TNF) etc. Additionally, saponin adjuvants, such as Stimulon™ (Cambridge Bioscience, Worcester, MA) may be used or particles generated therefrom such as ISCOMS (immunostimulating complexes). Furthermore, Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) may be

used. Alum and MF59 are preferred.

The immunogenic detoxified protein of the invention may be used as an adjuvant for a second antigen in an immunologically active composition for the treatment or vaccination of the human or animal body.

The immunogenic compositions (eg the antigen, pharmaceutically acceptable carrier and adjuvant) typically will contain diluents, such as water, saline, glycerol, ethanol, etc. Additionally, auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such vehicles.

Typically, the immunogenic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection may also be prepared. The preparation also may be emulsified or encapsulated in liposomes for enhanced adjuvant effect as discussed above under pharmaceutically acceptable carriers.

Immunogenic compositions used as vaccines comprise an immunologically effective amount of the antigenic polypeptides, as well as any other of the above-mentioned components, as needed. By "immunologically effective amount", it is meant that the administration of that amount to an individual, either in a single dose or as part of a series, is effective for treatment or prevention. This amount varies depending upon the health and physical condition of the individual to be treated, the taxonomic group of individual to be treated (eg., nonhuman primate, primate, etc.), the capacity of the individual's immune system to synthesize antibodies, the degree of protection desired, the formulation of the vaccine, the treating doctor's assessment of the medical situation, and other relevant factors. It is expected that the amount will fall in a relatively broad range that can be determined through

routine trials.

The immunogenic compositions are conventionally administered parenterally, eg. by injection either subcutaneously or
5 intramuscularly. Additional formulations suitable for other modes of administration include oral and pulmonary formulations, suppositories and transdermal applications. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunc-
10 tion with other immunoregulatory agents.

The term "recombinant polynucleotide" as used herein intends a polynucleotide of genomic, cDNA, semisynthetic, or synthetic origin which, by virtue of its origin or
15 manipulation: (1) is not associated with all or a portion of a polynucleotide with which it is associated in nature, (2) is linked to a polynucleotide other than that to which it is linked in nature, or (3) does not occur in nature.

20 The term "polynucleotide" as used herein refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, this term includes double- and single-stranded DNA and RNA. It
25 also includes known types of modifications, for example, labels which are known in the art, methylation, "caps", substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as, for example, those with uncharged linkages (eg.,
30 methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.) and with charged linkages (eg., phosphorothioates, phosphorodithioates, etc.), those containing pendant moieties, such as, for example proteins (including for eg., nucleases, toxins, antibodies, signal
35 peptides, poly-L-lysine, etc.), those with intercalators (eg., acridine, psoralen, etc.), those containing chelators (eg., metals, radioactive metals, boron, oxidative metals, etc.), those containing alkylators, those with modified

linkages (eg., alpha anomeric nucleic acids, etc.), as well as unmodified forms of the polynucleotide.

5 A "replicon" is any genetic element, eg., a plasmid, a chromosome, a virus, a cosmid, etc. that behaves as an autonomous unit of polynucleotide replication within a cell; i.e., capable of replication under its own control. This may include selectable markers.

10 A "vector" is a replicon in which another polynucleotide segment is attached, so as to bring about the replication and/or expression of the attached segment.

"Control sequence" refers to polynucleotide sequences which
15 are necessary to effect the expression of coding sequences to which they are ligated. The nature of such control sequences differs depending upon the host organism; in prokaryotes, such control sequences generally include promoter, ribosomal binding site, and transcription
20 termination sequence; in eukaryotes, generally, such control sequences include promoters and transcription termination sequence. The term "control sequences" is intended to include, at a minimum, all components whose presence is necessary for expression, and may also include additional
25 components whose presence is advantageous, for example, leader sequences and fusion partner sequences.

"Operably linked" refers to a juxtaposition wherein the components so described are in a relationship permitting
30 them to function in their intended manner. A control sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences.

35

An "open reading frame" (ORF) is a region of a polynucleotide sequence which encodes a polypeptide; this region may represent a portion of a coding sequence or a

total coding sequence.

A "coding sequence" is a polynucleotide sequence which is translated into a polypeptide, usually via mRNA, when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, cDNA, and recombinant polynucleotide sequences.

"PCR" refers to the technique of polymerase chain reaction as described in Saiki, et al., Nature 324:163 (1986); and Scharf et al., Science (1986) 233:1076-1078; and U.S. 4,683,195; and U.S. 4,683,202.

As used herein, x is "heterologous" with respect to y if x is not naturally associated with y in the identical manner; i.e., x is not associated with y in nature or x is not associated with y in the same manner as is found in nature.

"Homology" refers to the degree of similarity between x and y. The correspondence between the sequence from one form to another can be determined by techniques known in the art. For example, they can be determined by a direct comparison of the sequence information of the polynucleotide. Alternatively, homology can be determined by hybridization of the polynucleotides under conditions which form stable duplexes between homologous regions (for example, those which would be used prior to S_1 digestion), followed by digestion with single-stranded specific nuclease(s), followed by size determination of the digested fragments.

As used herein, the term "polypeptide" refers to a polymer of amino acids and does not refer to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide. This term also does not refer to or exclude post expression

modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid
5 (including, for example, unnatural amino acids, etc.), polypeptides with substituted linkages, as well as other modifications known in the art, both naturally occurring and non-naturally occurring.

10 A polypeptide or amino acid sequence "derived from" a designated nucleic acid sequence refers to a polypeptide having an amino acid sequence identical to that of a polypeptide encoded in the sequence, or a portion thereof wherein the portion consists of at least 3-5 amino acids,
15 and more preferably at least 8-10 amino acids, and even more preferably at least 11-15 amino acids, or which is immunologically identifiable with a polypeptide encoded in the sequence. This terminology also includes a polypeptide expressed from a designated nucleic acid sequence.

20

The protein may be used for producing antibodies, either monoclonal or polyclonal, specific to the protein. The methods for producing these antibodies are known in the art.

25 "Recombinant host cells", "host cells," "cells," "cell cultures," and other such terms denote, for example, microorganisms, insect cells, and mammalian cells, that can be, or have been, used as recipients for recombinant vector or other transfer DNA, and include the progeny of the
30 original cell which has been transformed. It is understood that the progeny of a single parental cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation. Examples for
35 mammalian host cells include Chinese hamster ovary (CHO) and monkey kidney (COS) cells.

Specifically, as used herein, "cell line," refers to a

population of cells capable of continuous or prolonged growth and division in vitro. Often, cell lines are clonal populations derived from a single progenitor cell. It is further known in the art that spontaneous or induced changes
5 can occur in karyotype during storage or transfer of such clonal populations. Therefore, cells derived from the cell line referred to may not be precisely identical to the ancestral cells or cultures, and the cell line referred to includes such variants. The term "cell lines" also includes
10 immortalized cells. Preferably, cell lines include nonhybrid cell lines or hybridomas to only two cell types.

As used herein, the term "microorganism" includes prokaryotic and eukaryotic microbial species such as
15 bacteria and fungi, the latter including yeast and filamentous fungi.

"Transformation", as used herein, refers to the insertion of an exogenous polynucleotide into a host cell, irrespective
20 of the method used for the insertion, for example, direct uptake, transduction, f-mating or electroporation. The exogenous polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host genome.

25 By "genomic" is meant a collection or library of DNA molecules which are derived from restriction fragments that have been cloned in vectors. This may include all or part of the genetic material of an organism.

30 By "cDNA" is meant a complementary DNA sequence that hybridizes to a complementary strand of DNA.

By "purified" and "isolated" is meant, when referring to a
35 polypeptide or nucleotide sequence, that the indicated molecule is present in the substantial absence of other biological macromolecules of the same type. The term "purified" as used herein preferably means at least 75% by

weight, more preferably at least 85% by weight, more preferably still at least 95% by weight, and most preferably at least 98% by weight, of biological macromolecules of the same type present (but water, buffers, and other small
5 molecules, especially molecules having a molecular weight of less than 1000, can be present).

Once the appropriate coding sequence is isolated, it can be expressed in a variety of different expression systems; for
10 example those used with mammalian cells, baculoviruses, bacteria, and yeast.

i. Mammalian Systems

15 Mammalian expression systems are known in the art. A mammalian promoter is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence (eg. structural gene) into mRNA. A promoter will have a transcription initiating
20 region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, usually located 25-30 base pairs (bp) upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian
25 promoter will also contain an upstream promoter element, usually located within 100 to 200 bp upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation [Sambrook et al. (1989) "Expression of Cloned
30 Genes in Mammalian Cells." In Molecular Cloning: A Laboratory Manual, 2nd ed.].

Mammalian viral genes are often highly expressed and have a broad host range; therefore sequences encoding mammalian
35 viral genes provide particularly useful promoter sequences. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter (Ad MLP), and herpes simplex virus promoter. In addition,

sequences derived from non-viral genes, such as the murine metallothionein gene, also provide useful promoter sequences. Expression may be either constitutive or regulated (inducible), depending on the promoter can be
5 induced with glucocorticoid in hormone-responsive cells.

The presence of an enhancer element (enhancer), combined with the promoter elements described above, will usually increase expression levels. An enhancer is a regulatory DNA
10 sequence that can stimulate transcription up to 1000-fold when linked to homologous or heterologous promoters, with synthesis beginning at the normal RNA start site. Enhancers are also active when they are placed upstream or downstream from the transcription initiation site, in either normal or
15 flipped orientation, or at a distance of more than 1000 nucleotides from the promoter [Maniatis et al. (1987) Science 236:1237; Alberts et al. (1989) Molecular Biology of the Cell, 2nd ed.]. Enhancer elements derived from viruses may be particularly useful, because they usually have a
20 broader host range. Examples include the SV40 early gene enhancer [Dijkema et al (1985) EMBO J. 4:761] and the enhancer/promoters derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus [Gorman et al. (1982b) Proc. Natl. Acad. Sci. 79:6777] and from human cytomegalovirus
25 [Boshart et al. (1985) Cell 41:521]. Additionally, some enhancers are regulatable and become active only in the presence of an inducer, such as a hormone or metal ion [Sassone-Corsi and Borelli (1986) Trends Genet. 2:215; Maniatis et al. (1987) Science 236:1237].

30

A DNA molecule may be expressed intracellularly in mammalian cells. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a
35 methionine, which is encoded by the ATG start codon. If desired, the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in mammalian cells. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The adenovirus tripartite leader is an example of a leader sequence that provides for secretion of a foreign protein in mammalian cells.

Usually, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-transcriptional cleavage and polyadenylation [Birnstiel et al. (1985) Cell 41:349; Proudfoot and Whitelaw (1988) "Termination and 3' end processing of eukaryotic RNA. In Transcription and splicing (ed. B.D. Hames and D.M. Glover); Proudfoot (1989) Trends Biochem. Sci. 14:105]. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Examples of transcription terminator/polyadenylation signals include those derived from SV40 [Sambrook et al (1989) "Expression of cloned genes in cultured mammalian cells." In Molecular Cloning: A Laboratory Manual].

Some genes may be expressed more efficiently when introns (also called intervening sequences) are present. Several cDNAs, however, have been efficiently expressed from vectors that lack splicing signals (also called splice donor and acceptor sites) [see eg., Gething and Sambrook (1981) Nature 293:620]. Introns are intervening noncoding sequences within

- a coding sequence that contain splice donor and acceptor sites. They are removed by a process called "splicing," following polyadenylation of the primary transcript [Nevins (1983) Annu. Rev. Biochem. 52:441; Green (1986) Annu. Rev.
5 Genet. 20:671; Padgett et al. (1986) Annu. Rev. Biochem. 55:1119; Krainer and Maniatis (1988) "RNA splicing." In Transcription and splicing (ed. B.D. Hames and D.M. Glover)].
- 10 Usually, the above described components, comprising a promoter, polyadenylation signal, and transcription termination sequence are put together into expression constructs. Enhancers, introns with functional splice donor and acceptor sites, and leader sequences may also be
15 included in an expression construct, if desired. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (eg., plasmids) capable of stable maintenance in a host, such as mammalian cells or bacteria. Mammalian replication systems include those derived from
20 animal viruses, which require trans-acting factors to replicate. For example, plasmids containing the replication systems of papovaviruses, such as SV40 [Gluzman (1981) Cell 23:175] or polyomavirus, replicate to extremely high copy number in the presence of the appropriate viral T antigen.
25 Additional examples of mammalian replicons include those derived from bovine papillomavirus and Epstein-Barr virus. Additionally, the replicon may have two replication systems, thus allowing it to be maintained, for example, in mammalian cells for expression and in a procaryotic host for cloning
30 and amplification. Examples of such mammalian-bacteria shuttle vectors include pMT2 [Kaufman et al. (1989) Mol. Cell. Biol. 9:946 and pHEBO [Shimizu et al. (1986) Mol. Cell. Biol. 6:1074].
- 35 The transformation procedure used depends upon the host to be transformed. Methods for introduction of heterologous polynucleotides into mammalian cells are known in the art and include dextran-mediated transfection, calcium phosphate

precipitation, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei.

5

Mammalian cell lines available as hosts for expression are known in the art and include many immortalized cell lines available from the American Type Culture Collection (ATCC), including but not limited to, Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, monkey kidney cells (COS), human hepatocellular carcinoma cells (eg., Hep G2), and a number of other cell lines.

ii. Baculovirus Systems

15 The polynucleotide encoding the protein can also be inserted into a suitable insect expression vector, and is operably linked to the control elements within that vector. Vector construction employs techniques which are known in the art.

20 Generally, the components of the expression system include a transfer vector, usually a bacterial plasmid, which contains both a fragment of the baculovirus genome, and a convenient restriction site for insertion of the heterologous gene or genes to be expressed; a wild type
25 baculovirus with a sequence homologous to the baculovirus-specific fragment in the transfer vector (this allows for the homologous recombination of the heterologous gene in to the baculovirus genome); and appropriate insect host cells and growth media.

30

After inserting the DNA sequence encoding the protein into the transfer vector, the vector and the wild type viral genome are transfected into an insect host cell where the vector and viral genome are allowed to recombine. The
35 packaged recombinant virus is expressed and recombinant plaques are identified and purified. Materials and methods for baculovirus/insect cell expression systems are commercially available in kit form from, inter alia,

Invitrogen, San Diego CA ("MaxBac" kit). These techniques are generally known to those skilled in the art and fully described in Summers and Smith, Texas Agricultural Experiment Station Bulletin No. 1555 (1987) (hereinafter 5 "Summers and Smith").

Prior to inserting the DNA sequence encoding the protein into the baculovirus genome, the above described components, comprising a promoter, leader (if desired), coding sequence 10 of interest, and transcription termination sequence, are usually assembled into an intermediate transplacement construct (transfer vector). This construct may contain a single gene and operably linked regulatory elements; multiple genes, each with its own set of operably linked 15 regulatory elements; or multiple genes, regulated by the same set of regulatory elements. Intermediate transplacement constructs are often maintained in a replicon, such as an extrachromosomal element (eg., plasmids) capable of stable maintenance in a host, such as a bacterium. The replicon 20 will have a replication system, thus allowing it to be maintained in a suitable host for cloning and amplification.

Currently, the most commonly used transfer vector for introducing foreign genes into AcNPV is pAc373. Many other 25 vectors, known to those of skill in the art, have also been designed. These include, for example, pVL985 (which alters the polyhedrin start codon from ATG to ATT, and which introduces a BamHI cloning site 32 basepairs downstream from the ATT; see Luckow and Summers, Virology (1989) 17:31.

30 The plasmid usually also contains the polyhedrin polyadenylation signal (Miller et al. (1988) Ann. Rev. Microbiol., 42:177) and a procaryotic ampicillin-resistance (amp) gene and origin of replication for selection and 35 propagation in *E. coli*.

Baculovirus transfer vectors usually contain a baculovirus promoter. A baculovirus promoter is any DNA sequence capable

of binding a baculovirus RNA polymerase and initiating the downstream (5' to 3') transcription of a coding sequence (eg. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A baculovirus transfer vector may also have a second domain called an enhancer, which, if present, is usually distal to the structural gene. Expression may be either regulated or constitutive.

Structural genes, abundantly transcribed at late times in a viral infection cycle, provide particularly useful promoter sequences. Examples include sequences derived from the gene encoding the viral polyhedron protein, Friesen et al., (1986) "The Regulation of Baculovirus Gene Expression," in: The Molecular Biology of Baculoviruses (ed. Walter Doerfler); EPO Publ. Nos. 127 839 and 155 476; and the gene encoding the p10 protein, Vlak et al., (1988), J. Gen. Virol. 69:765.

DNA encoding suitable signal sequences can be derived from genes for secreted insect or baculovirus proteins, such as the baculovirus polyhedrin gene (Carbonell et al. (1988) Gene, 73:409). Alternatively, since the signals for mammalian cell posttranslational modifications (such as signal peptide cleavage, proteolytic cleavage, and phosphorylation) appear to be recognized by insect cells, and the signals required for secretion and nuclear accumulation also appear to be conserved between the invertebrate cells and vertebrate cells, leaders of non-insect origin, such as those derived from genes encoding human α -interferon, Maeda et al., (1985), Nature 315:592; human gastrin-releasing peptide, Lebacqz-Verheyden et al., (1988), Molec. Cell. Biol. 8:3129; human IL-2, Smith et al., (1985) Proc. Nat'l Acad. Sci. USA, 82:8404; mouse IL-3, (Miyajima et al., (1987) Gene 58:273; and human

glucocerebrosidase, Martin et al. (1988) DNA, 7:99, can also be used to provide for secretion in insects.

A recombinant polypeptide or polyprotein may be expressed
5 intracellularly or, if it is expressed with the proper regulatory sequences, it can be secreted. Good intracellular expression of nonfused foreign proteins usually requires heterologous genes that ideally have a short leader sequence containing suitable translation initiation signals preceding
10 an ATG start signal. If desired, methionine at the N-terminus may be cleaved from the mature protein by in vitro incubation with cyanogen bromide.

Alternatively, recombinant polyproteins or proteins which
15 are not naturally secreted can be secreted from the insect cell by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provides for secretion of the foreign protein in insects. The leader sequence fragment usually encodes a signal
20 peptide comprised of hydrophobic amino acids which direct the translocation of the protein into the endoplasmic reticulum.

After insertion of the DNA sequence and/or the gene encoding
25 the expression product precursor of the protein, an insect cell host is co-transformed with the heterologous DNA of the transfer vector and the genomic DNA of wild type baculovirus -- usually by co-transfection. The promoter and transcription termination sequence of the construct will
30 usually comprise a 2-5kb section of the baculovirus genome. Methods for introducing heterologous DNA into the desired site in the baculovirus virus are known in the art. (See Summers and Smith supra; Ju et al. (1987); Smith et al., Mol. Cell. Biol. (1983) 3:2156; and Luckow and Summers
35 (1989)). For example, the insertion can be into a gene such as the polyhedrin gene, by homologous double crossover recombination; insertion can also be into a restriction enzyme site engineered into the desired baculovirus gene.

Miller et al., (1989), Bioessays 4:91. The DNA sequence, when cloned in place of the polyhedrin gene in the expression vector, is flanked both 5' and 3' by polyhedrin-specific sequences and is positioned downstream of the polyhedrin promoter.

The newly formed baculovirus expression vector is subsequently packaged into an infectious recombinant baculovirus. Homologous recombination occurs at low frequency (between about 1% and about 5%); thus, the majority of the virus produced after cotransfection is still wild-type virus. Therefore, a method is necessary to identify recombinant viruses. An advantage of the expression system is a visual screen allowing recombinant viruses to be distinguished. The polyhedrin protein, which is produced by the native virus, is produced at very high levels in the nuclei of infected cells at late times after viral infection. Accumulated polyhedrin protein forms occlusion bodies that also contain embedded particles. These occlusion bodies, up to 15 μm in size, are highly refractile, giving them a bright shiny appearance that is readily visualized under the light microscope. Cells infected with recombinant viruses lack occlusion bodies. To distinguish recombinant virus from wild-type virus, the transfection supernatant is plaqued onto a monolayer of insect cells by techniques known to those skilled in the art. Namely, the plaques are screened under the light microscope for the presence (indicative of wild-type virus) or absence (indicative of recombinant virus) of occlusion bodies. "Current Protocols in Microbiology" Vol. 2 (Ausubel et al. eds) at 16.8 (Supp. 10, 1990); Summers and Smith, supra; Miller et al. (1989).

Recombinant baculovirus expression vectors have been developed for infection into several insect cells. For example, recombinant baculoviruses have been developed for, inter alia: Aedes aegypti, Autographa californica, Bombyx mori, Drosophila melanogaster, Spodoptera frugiperda, and Trichoplusia ni (PCT Pub. No. WO 89/046699; Carbonell et

al., (1985) J. Virol. 56:153; Wright (1986) Nature 321:718; Smith et al., (1983) Mol. Cell. Biol. 3:2156; and see generally, Fraser, et al. (1989) In Vitro Cell. Dev. Biol. 25:225).

5

Cells and cell culture media are commercially available for both direct and fusion expression of heterologous polypeptides in a baculovirus/expression system; cell culture technology is generally known to those skilled in the art. See, eg., Summers and Smith [*supra*].

The modified insect cells may then be grown in an appropriate nutrient medium, which allows for stable maintenance of the plasmid(s) present in the modified insect host. Where the expression product gene is under inducible control, the host may be grown to high density, and expression induced. Alternatively, where expression is constitutive, the product will be continuously expressed into the medium and the nutrient medium must be continuously circulated, while removing the product of interest and augmenting depleted nutrients. The product may be purified by such techniques as chromatography, eg., HPLC, affinity chromatography, ion exchange chromatography, etc.; electrophoresis; density gradient centrifugation; solvent extraction, or the like. As appropriate, the product may be further purified, as required, so as to remove substantially any insect proteins which are also secreted in the medium or result from lysis of insect cells, so as to provide a product which is at least substantially free of host debris, eg., proteins, lipids and polysaccharides.

In order to obtain protein expression, recombinant host cells derived from the transformants are incubated under conditions which allow expression of the recombinant protein encoding sequence. These conditions will vary, dependent upon the host cell selected. However, the conditions are readily ascertainable to those of ordinary skill in the art, based upon what is known in the art.

iii. Bacterial Systems

Bacterial expression techniques are known in the art. A bacterial promoter is any DNA sequence capable of binding bacterial RNA polymerase and initiating the downstream (3') transcription of a coding sequence (eg. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site and a transcription initiation site. A bacterial promoter may also have a second domain called an operator, that may overlap an adjacent RNA polymerase binding site at which RNA synthesis begins. The operator permits negative regulated (inducible) transcription, as a gene repressor protein may bind the operator and thereby inhibit transcription of a specific gene. Constitutive expression may occur in the absence of negative regulatory elements, such as the operator. In addition, positive regulation may be achieved by a gene activator protein binding sequence, which, if present is usually proximal (5') to the RNA polymerase binding sequence. An example of a gene activator protein is the catabolite activator protein (CAP), which helps initiate transcription of the lac operon in *Escherichia coli* (*E. coli*) [Raibaud et al. (1984) Annu. Rev. Genet. 18:173]. Regulated expression may therefore be either positive or negative, thereby either enhancing or reducing transcription.

Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences derived from sugar metabolizing enzymes, such as galactose, lactose (lac) [Chang et al. (1977) Nature 198:1056], and maltose. Additional examples include promoter sequences derived from biosynthetic enzymes such as tryptophan (trp) [Goeddel et al. (1980) Nuc. Acids Res. 8:4057; Yelverton et al. (1981) Nucl. Acids Res. 9:731; U.S. Patent No. 4,738,921; EPO Publ. Nos. 036 776 and 121 775].

The g-laotamase (bla) promoter system [Weissmann (1981) "The cloning of interferon and other mistakes." In Interferon 3 (ed. I. Gresser)], bacteriophage lambda PL [Shimatake et al. (1981) Nature 292:128] and T5 [U.S. Patent No. 4,689,406]
5 promoter systems also provide useful promoter sequences.

In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. For example, transcription activation sequences of one bacterial or
10 bacteriophage promoter may be joined with the operon sequences of another bacterial or bacteriophage promoter, creating a synthetic hybrid promoter [U.S. Patent No. 4,551,433]. For example, the tac promoter is a hybrid trp-lac promoter comprised of both trp promoter and lac
15 operon sequences that is regulated by the lac repressor [Amann et al. (1983) Gene 25:167; de Boer et al. (1983) Proc. Natl. Acad. Sci. 80:21]. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA
20 polymerase and initiate transcription. A naturally occurring promoter of non-bacterial origin can also be coupled with a compatible RNA polymerase to produce high levels of expression of some genes in prokaryotes. The bacteriophage T7 RNA polymerase/promoter system is an example of a coupled
25 promoter system [Studier et al. (1986) J. Mol. Biol. 189:113; Tabor et al. (1985) Proc Natl. Acad. Sci. 82:1074]. In addition, a hybrid promoter can also be comprised of a bacteriophage promoter and an E. coli operator region (EPO Publ. No. 267 851).

30

In addition to a functioning promoter sequence, an efficient ribosome binding site is also useful for the expression of foreign genes in prokaryotes. In E. coli, the ribosome binding site is called the Shine-Dalgarno (SD) sequence and
35 includes an initiation codon (ATG) and a sequence 3-9 nucleotides in length located 3-11 nucleotides upstream of the initiation codon [Shine et al. (1975) Nature 254:34]. The SD sequence is thought to promote binding of mRNA to the

- ribosome by the pairing of bases between the SD sequence and the 3' end of E. coli 16S rRNA [Steitz et al. (1979) "Genetic signals and nucleotide sequences in messenger RNA." In Biological Regulation and Development: Gene Expression (ed. R.F. Goldberger)]. To express eukaryotic genes and prokaryotic genes with weak ribosome-binding site [Sambrook et al. (1989) "Expression of cloned genes in *Escherichia coli*." In Molecular Cloning: A Laboratory Manual].
- 10 A DNA molecule may be expressed intracellularly. A promoter sequence may be directly linked with the DNA molecule, in which case the first amino acid at the N-terminus will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be
- 15 cleaved from the protein by in vitro incubation with cyanogen bromide or by either in vivo or in vitro incubation with a bacterial methionine N-terminal peptidase (EPO Publ. No. 219 237).
- 20 Fusion proteins provide an alternative to direct expression. Usually, a DNA sequence encoding the N-terminal portion of an endogenous bacterial protein, or other stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two
- 25 amino acid sequences. For example, the bacteriophage lambda cell gene can be linked at the 5' terminus of a foreign gene and expressed in bacteria. The resulting fusion protein preferably retains a site for a processing enzyme (factor Xa) to cleave the bacteriophage protein from the foreign
- 30 gene [Nagai et al. (1984) Nature 309:810]. Fusion proteins can also be made with sequences from the lacZ [Jia et al. (1987) Gene 60:197], trpE [Allen et al. (1987) J. Biotechnol. 5:93; Makoff et al. (1989) J. Gen. Microbiol. 135:11], and Chey [EPO Publ. No. 324 647] genes. The DNA
- 35 sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a

processing enzyme (eg. ubiquitin specific processing-protease) to cleave the ubiquitin from the foreign protein. Through this method, native foreign protein can be isolated [Miller et al. (1989) Bio/Technology 7:698].

5

Alternatively, foreign proteins can also be secreted from the cell by creating chimeric DNA molecules that encode a fusion protein comprised of a signal peptide sequence fragment that provides for secretion of the foreign protein
10 in bacteria [U.S. Patent No. 4,336,336]. The signal sequence fragment usually encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. The protein is either secreted into the growth media (gram-positive bacteria) or into the
15 periplasmic space, located between the inner and outer membrane of the cell (gram-negative bacteria). Preferably there are processing sites, which can be cleaved either in vivo or in vitro encoded between the signal peptide fragment and the foreign gene.

20

DNA encoding suitable signal sequences can be derived from genes for secreted bacterial proteins, such as the E. coli outer membrane protein gene (ompA) [Masui et al. (1983), in: Experimental Manipulation of Gene Expression; Ghayeb et al.
25 (1984) EMBO J. 3:2437] and the E. coli alkaline phosphatase signal sequence (phoA) [Oka et al. (1985) Proc. Natl. Acad. Sci. 82:7212]. As an additional example, the signal sequence of the alpha-amylase gene from various Bacillus strains can be used to secrete heterologous proteins from B. subtilis
30 [Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. No. 244 042].

Usually, transcription termination sequences recognized by bacteria are regulatory regions located 3' to the
35 translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded by the DNA. Transcription termination

sequences frequently include DNA sequences of about 50 nucleotides capable of forming stem loop structures that aid in terminating transcription. Examples include transcription termination sequences derived from genes with strong
5 promoters, such as the trp gene in E. coli as well as other biosynthetic genes.

Usually, the above described components, comprising a promoter, signal sequence (if desired), coding sequence of
10 interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often maintained in a replicon, such as an extrachromosomal element (eg., plasmids) capable of stable maintenance in a host, such as bacteria. The replicon will
15 have a replication system, thus allowing it to be maintained in a procaryotic host either for expression or for cloning and amplification. In addition, a replicon may be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to
20 about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably contain at least about 10, and more preferably at least about 20 plasmids. Either a high or low copy number vector may be selected, depending upon the effect of the vector and
25 the foreign protein on the host.

Alternatively, the expression constructs can be integrated into the bacterial genome with an integrating vector. Integrating vectors usually contain at least one sequence
30 homologous to the bacterial chromosome that allows the vector to integrate. Integrations appear to result from recombinations between homologous DNA in the vector and the bacterial chromosome. For example, integrating vectors constructed with DNA from various *Bacillus* strains integrate
35 into the *Bacillus* chromosome (EPO Publ. No. 127 328). Integrating vectors may also be comprised of bacteriophage or transposon sequences.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the selection of bacterial strains that have been transformed. Selectable markers can be expressed in the bacterial host
5 and may include genes which render bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin (neomycin), and tetracycline [Davies et al. (1978) Annu. Rev. Microbiol. 32:469]. Selectable markers may also include biosynthetic genes, such as those in the histidine,
10 tryptophan, and leucine biosynthetic pathways.

Alternatively, some of the above described components can be put together in transformation vectors. Transformation vectors are usually comprised of a selectable market that is
15 either maintained in a replicon or developed into an integrating vector, as described above.

Expression and transformation vectors, either extra-chromosomal replicons or integrating vectors, have been
20 developed for transformation into many bacteria. For example, expression vectors have been developed for, inter alia, the following bacteria: *Bacillus subtilis* [Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541],
25 *Escherichia coli* [Shimatake et al. (1981) Nature 292:128; Amann et al. (1985) Gene 40:183; Studier et al. (1986) J. Mol. Biol. 189:113; EPO Publ. Nos. 036 776, 136 829 and 136 907], *Streptococcus cremoris* [Powell et al. (1988) Appl. Environ. Microbiol. 54:655]; *Streptococcus lividans* [Powell
30 et al. (1988) Appl. Environ. Microbiol. 54:655], *Streptomyces lividans* [U.S. Patent No. 4,745,056].

Methods of introducing exogenous DNA into bacterial hosts are well-known in the art, and usually include either the
35 transformation of bacteria treated with CaCl_2 or other agents, such as divalent cations and DMSO. DNA can also be introduced into bacterial cells by electroporation. Transformation procedures usually vary with the bacterial

species to be transformed. See eg., [Masson et al. (1989) FEMS Microbiol. Lett. 60:273; Palva et al. (1982) Proc. Natl. Acad. Sci. USA 79:5582; EPO Publ. Nos. 036 259 and 063 953; PCT Publ. No. WO 84/04541, Bacillus], [Miller et al. (1988) Proc. Natl. Acad. Sci. 85:856; Wang et al. (1990) J. Bacteriol. 172:949, Campylobacter], [Cohen et al. (1973) Proc. Natl. Acad. Sci. 69:2110; Dower et al. (1988) Nucleic Acids Res. 16:6127; Kushner (1978) "An improved method for transformation of Escherichia coli with ColE1-derived plasmids. In Genetic Engineering: Proceedings of the International Symposium on Genetic Engineering (eds. H.W. Boyer and S. Nicosia); Mandel et al. (1970) J. Mol. Biol. 53:159; Taketo (1988) Biochim. Biophys. Acta 949:318; Escherichia], [Chassy et al. (1987) FEMS Microbiol. Lett. 44:173 Lactobacillus]; [Fiedler et al. (1988) Anal. Biochem 170:38, Pseudomonas]; [Augustin et al. (1990) FEMS Microbiol. Lett. 66:203, Staphylococcus], [Barany et al. (1980) J. Bacteriol. 144:698; Harlander (1987) "Transformation of Streptococcus lactis by electroporation, in: Streptococcal Genetics (ed. J. Ferretti and R. Curtiss III); Perry et al. (1981) Infec. Immun. 32:1295; Powell et al. (1988) Appl. Environ. Microbiol. 54:655; Somkuti et al. (1987) Proc. 4th Eur. Cong. Biotechnology 1:412, Streptococcus].

25

iv. Yeast Expression

Yeast expression systems are also known to one of ordinary skill in the art. A yeast promoter is any DNA sequence capable of binding yeast RNA polymerase and initiating the downstream (3') transcription of a coding sequence (eg. structural gene) into mRNA. A promoter will have a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region usually includes an RNA polymerase binding site (the "TATA Box") and a transcription initiation site. A yeast promoter may also have a second domain called an upstream activator sequence (UAS), which,

if present, is usually distal to the structural gene. The UAS permits regulated (inducible) expression. Constitutive expression occurs in the absence of a UAS. Regulated expression may be either positive or negative, thereby
5 either enhancing or reducing transcription.

Yeast is a fermenting organism with an active metabolic pathway, therefore sequences encoding enzymes in the metabolic pathway provide particularly useful promoter
10 sequences. Examples include alcohol dehydrogenase (ADH) (EPO Publ. No. 284 044), enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase (GAP or GAPDH), hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, and pyruvate
15 kinase (PyK) (EPO Publ. No. 329 203). The yeast PHO5 gene, encoding acid phosphatase, also provides useful promoter sequences [Myanohara et al. (1983) Proc. Natl. Acad. Sci. USA 80:1].

20 In addition, synthetic promoters which do not occur in nature also function as yeast promoters. For example, UAS sequences of one yeast promoter may be joined with the transcription activation region of another yeast promoter, creating a synthetic hybrid promoter. Examples of such
25 hybrid promoters include the ADH regulatory sequence linked to the GAP transcription activation region (U.S. Patent Nos. 4,876,197 and 4,880,734). Other examples of hybrid promoters include promoters which consist of the regulatory sequences of either the ADH2, GAL4, GAL10, OR PHO5 genes, combined
30 with the transcriptional activation region of a glycolytic enzyme gene such as GAP or PyK (EPO Publ. No. 164 556). Furthermore, a yeast promoter can include naturally occurring promoters of non-yeast origin that have the ability to bind yeast RNA polymerase and initiate
35 transcription. Examples of such promoters include, inter alia, [Cohen et al. (1980) Proc. Natl. Acad. Sci. USA 77:1078; Henikoff et al. (1981) Nature 283:835; Hollenberg et al. (1981) Curr. Topics Microbiol. Immunol. 96:119;

- Hollenberg et al. (1979) "The Expression of Bacterial Antibiotic Resistance Genes i the Yeast *Saccharomyces cerevisiae*," in: Plasmids of Medical, Environmental and Commercial Importance (eds. K>N> Timmis and A. Puhler);
- 5 Mercerau-Puigalon et al. (1980) Gene 11:163; Panthier et al. (1980) Curr. Genet. 2:109;].

A DNA molecule may be expressed intracellularly in yeast. A promoter sequence may be directly linked with the DNA
10 molecule, in which case the first amino acid at the N-terminus of the recombinant protein will always be a methionine, which is encoded by the ATG start codon. If desired, methionine at the N-terminus may be cleaved from the protein by in vitro incubation with cyanogen bromide.

15

Fusion proteins provide an alternative for yeast expression systems, as well as in mammalian, baculovirus, and bacterial expression systems. Usually, a DNA sequence encoding the N-terminal portion of an endogenous yeast protein, or other
20 stable protein, is fused to the 5' end of heterologous coding sequences. Upon expression, this construct will provide a fusion of the two amino acid sequences. For example, the yeast or human superoxide dismutase (SOD) gene, can be linked at the 5' terminus of a foreign gene and
25 expressed in yeast. The DNA sequence at the junction of the two amino acid sequences may or may not encode a cleavable site. See eg., EPO Publ. No. 196 056. Another example is a ubiquitin fusion protein. Such a fusion protein is made with the ubiquitin region that preferably retains a site for a
30 processing enzyme (eg. ubiquitin-specific processing protease) to cleave the ubiquitin from the foreign protein. Through this method, therefore, native foreign protein can be isolated (see, eg., PCT Publ. No. WO 88/024066).

35 Alternatively, foreign proteins can also be secreted from the cell into the growth media by creating chimeric DNA molecules that encode a fusion protein comprised of a leader sequence fragment that provide for secretion in yeast of the

foreign protein. Preferably, there are processing sites encoded between the leader fragment and the foreign gene that can be cleaved either in vivo or in vitro. The leader sequence fragment usually encodes a signal peptide comprised
5 of hydrophobic amino acids which direct the secretion of the protein from the cell.

DNA encoding suitable signal sequences can be derived from genes for secreted yeast proteins, such as the yeast
10 invertase gene (EPO Publ. No. 012 873; JPO Publ. No. 62,096,086) and the A-factor gene (U.S. Patent No. 4,588,684). Alternatively, leaders of non-yeast origin, such as an interferon leader, exist that also provide for secretion in yeast (EPO Publ. No. 060 057).

15

A preferred class of secretion leaders are those that employ a fragment of the yeast alpha-factor gene, which contains both a "pre" signal sequence, and a "pro" region. The types of alpha-factor fragments that can be employed include the
20 full-length pre-pro alpha factor leader (about 83 amino acid residues) as well as truncated alpha-factor leaders (usually about 25 to about 50 amino acid residues) (U.S. Patent Nos. 4,546,083 and 4,870,008; EPO Publ. No. 324 274). Additional leaders employing an alpha-factor leader fragment that
25 provides for secretion include hybrid alpha-factor leaders made with a presequence of a first yeast, but a pro-region from a second yeast alphafactor. (See eg., PCT Publ. No. WO 89/02463.)

30 Usually, transcription termination sequences recognized by yeast are regulatory regions located 3' to the translation stop codon, and thus together with the promoter flank the coding sequence. These sequences direct the transcription of an mRNA which can be translated into the polypeptide encoded
35 by the DNA. Examples of transcription terminator sequence and other yeast-recognized termination sequences, such as those coding for glycolytic enzymes.

Usually, the above described components, comprising a promoter, leader (if desired), coding sequence of interest, and transcription termination sequence, are put together into expression constructs. Expression constructs are often
5 maintained in a replicon, such as an extrachromosomal element (eg., plasmids) capable of stable maintenance in a host, such as yeast or bacteria. The replicon may have two replication systems, thus allowing it to be maintained, for example, in yeast for expression and in a procaryotic host
10 for cloning and amplification. Examples of such yeast-bacteria shuttle vectors include YEp24 [Botstein et al. (1979) Gene 8:17-24], pCl/1 [Brake et al. (1984) Proc. Natl. Acad. Sci. USA 81:4642-4646], and YRp17 [Stinchcomb et al. (1982) J. Mol. Biol. 158:157]. In addition, a replicon may
15 be either a high or low copy number plasmid. A high copy number plasmid will generally have a copy number ranging from about 5 to about 200, and usually about 10 to about 150. A host containing a high copy number plasmid will preferably have at least about 10, and more preferably at
20 least about 20. Enter a high or low copy number vector may be selected, depending upon the effect of the vector and the foreign protein on the host. See eg., Brake et al., supra.

Alternatively, the expression constructs can be integrated
25 into the yeast genome with an integrating vector. Integrating vectors usually contain at least one sequence homologous to a yeast chromosome that allows the vector to integrate, and preferably contain two homologous sequences flanking the expression construct. Integrations appear to
30 result from recombinations between homologous DNA in the vector and the yeast chromosome [Orr-Weaver et al. (1983) Methods in Enzymol. 101:228-245]. An integrating vector may be directed to a specific locus in yeast by selecting the appropriate homologous sequence for inclusion in the vector.
35 See Orr-Weaver et al., supra. One or more expression construct may integrate, possibly affecting levels of recombinant protein produced [Rine et al. (1983) Proc. Natl. Acad. Sci. USA 80:6750]. The chromosomal sequences included

in the vector can occur either as a single segment in the vector, which results in the integration of the entire vector, or two segments homologous to adjacent segments in the chromosome and flanking the expression construct in the
5 vector, which can result in the stable integration of only the expression construct.

Usually, extrachromosomal and integrating expression constructs may contain selectable markers to allow for the
10 selection of yeast strains that have been transformed. Selectable markers may include biosynthetic genes that can be expressed in the yeast host, such as ADE2, HIS4, LEU2, TRP1, and ALG7, and the G418 resistance gene, which confer resistance in yeast cells to tunicamycin and G418,
15 respectively. In addition, a suitable selectable marker may also provide yeast with the ability to grow in the presence of toxic compounds, such as metal. For example, the presence of CUP1 allows yeast to grow in the presence of copper ions [Butt et al. (1987) Microbiol. Rev. 51:351].

20 Alternatively, some of the above described components can be put together into transformation vectors. Transformation vectors are usually comprised of a selectable marker that is either maintained in a replicon or developed into an
25 integrating vector, as described above.

Expression and transformation vectors, either extrachromosomal replicons or integrating vectors, have been developed for transformation into many yeasts. For example,
30 expression vectors have been developed for, inter alia, the following yeasts: *Candida albicans* [Kurtz, et al. (1986) Mol. Cell. Biol. 6:142], *Candida maltose* [Kunze, et al. (1985) J. Basic Microbiol. 25:141], *Hansenula polymorpha* [Gleeson, et al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al.
35 (1986) Mol. Gen. Genet. 202:302], *Kluyveromyces fragilis* [Das, et al. (1984) J. Bacteriol. 158:1165], *Kluyveromyces lactis* [De Louvencourt et al. (1983) J. Bacteriol. 154:737; Van den Berg et al. (1990) Bio/Technology 8:135], *Pichia*

guillerimondii [Kunze et al. (1985) J. Basic Microbiol. 25:141], *Pichia pastoris* [Cregg, et al. (1985) Mol. Cell. Biol. 5:3376; U.S. Patent Nos. 4,837,148 and 4,929,555], *Saccharomyces cerevisiae* [Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163], *Schizosaccharomyces pombe* [Beach and Nurse (1981) Nature 300:706], and *Yarrowia lipolytica* [Davidow, et al. (1985) Curr. Genet. 10:380471 Gaillardin, et al. (1985) Curr. Genet. 10:49].

10

Methods of introducing exogenous DNA into yeast hosts are well-known in the art, and usually include either the transformation of spheroplasts or of intact yeast cells treated with alkali cations. Transformation procedures usually vary with the yeast species to be transformed. See eg., [Kurtz et al. (1986) Mol. Cell. Biol. 6:142; Kunze et al. (1985) J. Basic Microbiol. 25:141; Candida]; [Gleeson et al. (1986) J. Gen. Microbiol. 132:3459; Roggenkamp et al. (1986) Mol. Gen. Genet. 202:302; Hansenula]; [Das et al. (1984) J. Bacteriol. 158:1165; De Louvencourt et al. (1983) J. Bacteriol. 154:1165; Van den Berg et al. (1990) Bio/Technology 8:135; Kluyveromyces]; [Cregg et al. (1985) Mol. Cell. Biol. 5:3376; Kunze et al. (1985) J. Basic Microbiol. 25:141; U.S. Patent Nos. 4,837,148 and 4,929,555; *Pichia*]; [Hinnen et al. (1978) Proc. Natl. Acad. Sci. USA 75:1929; Ito et al. (1983) J. Bacteriol. 153:163 *Saccharomyces*]; [Beach and Nurse (1981) Nature 300:706; *Schizosaccharomyces*]; [Davidow et al. (1985) Curr. Genet. 10:39; Gaillardin et al. (1985) Curr. Genet. 10:49; *Yarrowia*].

1. Preparation of LT-A72R mutant

a. Source of LT DNA

35 The 1.5 kb *SmaI-EcoRI* fragment from plasmid pEWD299, containing the LT-A gene and the LTpromoter region [Pronk et al. (1985) J. Biol. Chem. 260:13580; Spicer et al. (1981) Proc. Natl. Acad. Sci. USA 78:50] was subcloned into the

Bluescript KS vector (Stratagene). The resultant vector, termed BS-LT-A, was used for site-directed mutagenesis [Zoller & Smith (1982) *Nucl. Acid. Res.* 10:6487)].

5 **b. Methods of mutation**

Site-directed mutagenesis was performed according to the method of Zoller & Smith [*supra*] on single-stranded DNA of BS-LT-A vector. The oligonucleotide used:

5'GCTCACTTACGTGGACAGTCT³' (oligoLT-A72R)

10 mutates the codon for Ala-72 (GCA) to an Arg codon (CGT).

The mutated *Sma*I-*Eco*RI fragment containing the mutated codon was ligated with the 0.57 kb *Eco*RI-*Hind*III fragment [Pronk et al.; Spicer et al.] containing the gene coding for LT-B
15 and cloned into *Bluescript KS* vector (Stratagene) to generate vector BS-LTA72R.

c. Expression and purification of the LT-A72R mutant.

E.coli were transformed with BS-LTA72R vector and grown in
20 LB broth in a 5 litre fermenter. The mutant protein LT-A72R was purified from the periplasm [Pronk et al.; Magagnoli et al. (1996) *Infect Immun* 64:5434] using controlled pore glass (CPG 350, Serva) and A5M Agarose columns and then by gel filtration using Sephacryl S-200™.

25

An aliquot of the purified material was analysed on a Superdex 200HR column. Figure 1 shows the elution profile of LT-A72R in comparison with wild-type LT and mutant LTK63 (Ser-63-Lys mutant - see W093/13202 and also Pizza et al.
30 (1994) *J.Exp. Med.* 6:2147).

LT-A72R elutes as a single peak corresponding to the fully-assembled holotoxin, with a profile identical to that of wild-type LT and LTK63. This demonstrates that the A72R
35 mutation does not alter the structure of the A subunit, that it is efficiently exported to the periplasm, and correctly assembled as AB₅. 100μl aliquots of A72R stored at 4°C were analysed in the same way every two months for a year and no

change in elution profile was detected.

2. Physical characterisation

5 a. Trypsin digestion

45µg toxin (LT, LT-A72R, LTK63) was treated with 9µg trypsin in a final volume of 150µl 10mM Tris, pH 7.5, at 37°C. 30µl samples were collected after 5 and 30 minutes incubation and the reaction was stopped with 3.6µg trypsin inhibitor. 10µl
10 4xconcentrated electrophoresis sample buffer was added to each sample and the mixture was heated to 95°C for 10 minutes. Proteins were loaded onto 15% SDS minigels and either stained with Coomassie brilliant blue R-250 or transferred onto a nitrocellulose membrane, which was
15 subsequently incubated with a rabbit anti-LT polyclonal serum at a dilution of 1:300.

Figure 2 shows the results of this Western blot analysis. After 5 minutes incubation, trypsin causes almost immediate
20 nicking of the A subunit into A1 and A2 domains, and nicking is complete by 30 minutes. No sensitivity differences were detected between LT, LT-A72R, or LTK63.

b. ADP-ribosylation of polyarginine

25 LT (wild-type and mutant) was analysed as described by Lai et al. [BBRC 102:1021, 1981] and the results are shown in Figure 3.

The ADP-ribosylation activity of LT-A72R is at least 2
30 orders of magnitude lower than that of wild-type LT: the minimal concentration to obtain a detectable activity was 0.5µg for LT, and 85µg for LT-A72R. In contrast, LTK63 is totally devoid of activity.

35 c. Toxicity

In vitro toxicity was assessed by following the morphological changes caused by LT on Y1 adrenal cells [Donta et al. (1973) Science 183:334]. The assay was

performed in microtitre plates using 50000 cells/well with twofold dilutions of LT, LT-A72R and LTK63, starting from 80pg/well for wild-type and 40µg/well for mutants. Morphological changes were read after 48 hours incubation.

5

In vivo toxicity was assessed using an ileal loop assay [De (1959) *Nature* 183:1533]. Two New Zealand adult rabbits (ca. 2.5 kg) were used for each assay and 1ml samples of toxin (wild-type, LT-A72R, LTK63) were injected into each loop at various concentrations, with a control loop receiving 1ml PBS. After 18-20 hours, the volume of liquid accumulated in each loop was measured with a syringe and the length of each loop was measured again. The results, from 4-6 repeats, expressed as volume of liquid per unit length of the loop (ml/cm) are shown in Figure 5.

In agreement with the ADP-ribosylation results, LT-A72R had 10^{-5} toxicity of wild-type LT on Y1 cells (Figure 4) and was approximately 20x less toxic than wild-type in the ileal loop assay (Figure 5). As previously reported, LTK63 was completely non-toxic by both assays.

3. Immunological characterisation

25 a. Mucosal adjuvanticity

The adjuvanticity of LT-A72R was tested using the protocol of Douce et al. [*PNAS* 92:1644-1648 (1995)]. Groups of ten BALB/c mice (female, 4-6 weeks old) were immunised intranasally with 1µg toxin (LT, LT-A72R, LTB, or LK-K63) and 10µg OVA. The animals were lightly anaesthetised and immunised on day 0, 21 and 35 with a 15µl volume per nostril. Immune responses were followed in serum samples taken on days 0, 20, 34 and 52. The animals were sacrificed and nasal lavages were performed by repeated flushing and aspiration of 1ml PBS containing 0.1% BSA.

LT-specific antibodies were measured using a GM1 capture ELISA. Each well on 96-well plates was first coated with

150ng GM1 ganglioside by overnight incubation at 4°C. Wells were then washed three times with PBS (+0.05% Tween-20) and 50ng toxin was added to each well. Plates were incubated at 2 hours at 37°C. OVA-specific antibodies were assessed by coating each well with 60µg/ml OVA and incubated overnight at 4°C.

The plates were washed and wells saturated with PBS (+1% BSA) for 1 hour at 37°C. Sera from individual mice were tested starting from a dilution of 1:50 in PBS; nasal lavages were tested starting from undiluted. Plates were incubated at 37°C for 2 hours. Specific Ig were measured using horseradish peroxidase-conjugated rabbit anti-mouse Ig. Antibodies were then revealed by adding o-phenylenediamine as a substrate. After 10 minutes the reaction was blocked by adding 12.5% H₂SO₄.

IgG subclasses were determined with IgG1, IgG2a, IgG2b, or IgG3 biotinylated antibodies. Peroxidase-streptavidin was then added to each well (1:1000) and the plates were incubated at 37°C for 1 hour. Bound antibody was visualised as above.

Absorbances were read at 490nm and ELISA titres were determined arbitrarily as the reciprocal of the last dilution which gave an OD₄₉₀ ≥ 0.3 above the level measured in pre-immune sera.

Titres of specific IgA in the sera and in the mucosal lavages were measured using biotin-conjugated goat anti-mouse IgA α chain specific antibody, followed by streptavidin-peroxidase. Bound antibodies were revealed using the OPD substrate as described above. ELISA titres were determined arbitrarily as the reciprocal of the last dilution which gave an OD₄₉₀ ≥ 0.2 above that of the non-immunised controls.

Values were always normalised using a positive control

sample in each plate.

As shown in Figure 6, wild-type LT and LT-A72R induced the highest anti-OVA antibody response. LTK63 induced an intermediate level, and LTB gave a low response. The antigen-specific antibody response was detectable after a single immunisation for wild-type LT and the A72R mutant, whereas (as previously described) LTK63 required at least two immunisations to induce serum anti-OVA antibodies.

10

OVA-specific IgG isotypes were measured in pools of sera of the last bleeding (Figure 7). High titres of anti-OVA IgG1, IgG2a and IgG2b were induced in the groups receiving wild-type LT or the A72R mutant as an adjuvant, although the dominant isotype with the mutant was IgG2a. OVA-specific IgG3 antibodies were never detectable. LTB was not a good adjuvant.

In all experiments, OVA-specific IgA was never detected after just 1 or two intranasal immunisations. Following a third immunisation, however, a serum IgA response was detected with wild-type LT and with the A72R and K63 mutants, but not with LTB (Figure 8A). A similar pattern was seen at the mucosal level (Figure 8B), and after three immunisations OVA-specific IgA was found in the nasal washes of the wild-type group and the A72R and K63 mutant groups, but not in the control or LTB groups.

Serum anti-LT antibody responses are shown in Figure 9. The mice mounted responses after the first immunisation, which was significantly boosted by the second. The A72R mutant was more immunogenic than the non-toxic K63 mutant, and both were much more immunogenic than LTB.

35 b. OVA-driven proliferative response

14-20 days after 2 or 3 intranasal immunisations with OVA+LT (wild-type or mutant), 2 or 3 mice per group were sacrificed and spleens removed. Spleen cell suspensions were obtained

and resuspended in complete DMEM (10% FCS, 2mM L-glutamine, 15mM Hepes, 100U penicillin/streptomycin, 50mM 2-mercapto-ethanol). 2×10^5 spleen cells/well were seeded in U-bottomed 96-well plates and cultured in the presence of different concentrations of OVA for 5 days. [^3H]-thymidine was added (1 μCi /well) 16 hours before the end of culture. Cells were then harvested with a cell harvester, and [^3H]-thymidine incorporation was evaluated by liquid scintillation counting (Figure 10).

10

It is clear that intranasal co-administration of antigen with wild-type or with A72R or K63 mutants induced a priming of OVA-specific T cells *in vivo*, which was much stronger than that detectable after immunisation with OVA alone or with OVA+LTB.

15

c. *In vivo* challenge

The LD₅₀ for LT was determined by inoculating groups of 10 BALB/c mice (female, 9 weeks old) intraperitoneally with LT (12.5 μg , 25 μg , 50 μg , or 100 μg) or PBS. After 7 days of observation, LD₅₀ was determined as 20.4 μg .

20

Four week old BALB/c mice were immunised intranasally at day 0 and 21 with 1 μg toxin (LT, LT-A72R, LT-K63, or LTB) and challenged with LT (2xLD₅₀) at day 35. They were observed for death for 7 days. Sera were collected at days 0, 20 and 35 and anti-LT titres analysed by ELISA (as above).

25

All mice immunised with wild-type or mutant LT survived to the challenge, whereas only 30% of those receiving LTB survived. All mice in the control group died (Figure 11A).

30

Sera of mice immunised with wild-type LT, or with the A72R or K63 mutants, contained very high and comparable levels of anti-LT antibodies (Figure 11B). In mice receiving LTB, however, the titres were 10-20x lower. The three LTB mice who survived to the LT challenge (open circles) had significantly higher titres than those in the dead animals.

35

4. Conclusions

ADP-ribosylation activity is not necessary for the adjuvanticity of LT, but the presence of low levels of enzymatic activity may be useful to induce a faster and higher immune response to co-administered antigens.

The A72R mutant of LT is an effective mucosal adjuvant. In addition, the mutant retains the immunogenicity of wild-type LT and is able to induce protective immunity against LT. It may therefore also be useful for anti-diarrhoea vaccination.

It will, of course, be understood that the invention is described above by way of example only and modifications may be made within the scope and spirit of the invention.

CLAIMS

1. An immunogenic detoxified protein comprising the amino acid sequence of subunit A of an *E.coli* heat labile toxin (LT-A), or a fragment thereof, wherein at least amino acid Ala-72 in said sequence or fragment is mutated.
2. An immunogenic detoxified protein according to claim 1, wherein Ala-72 is mutated by substitution.
3. An immunogenic detoxified protein according to claim 2, wherein Ala-72 is substituted with an arginine residue.
4. An immunogenic composition comprising an immunogenic detoxified protein according to any one of claims 1 to 3 and a pharmaceutically acceptable carrier.
5. An immunogenic composition according to claim 4 further comprising an adjuvant.
6. An immunogenic composition according to claim 4 or claim 5 further comprising a second immunogenic antigen.
7. The use of a detoxified protein according to any one of claims 1 to 3 as an adjuvant.
8. A method of vaccinating a mammal against an enterotoxigenic strain of *E.coli* comprising administering an effective amount of an immunogenic detoxified protein according to any one of claims 1 to 3.
9. A DNA sequence encoding an immunogenic detoxified protein according to any one of claims 1 to 3.
10. A vector comprising a DNA sequence according to claim 9.
11. A host cell transformed with the vector of claim 10.

12. A process for the production of an immunogenic detoxified protein according to any one of claims 1 to 3 comprising culturing a host cell according to claim 11.
- 5 13. A process for the production of a DNA according to claim 9 comprising the steps of subjecting a DNA encoding a wild-type LT-A or a fragment thereof to site-directed mutagenesis.
- 10 14. A process for the formulation of an immunogenic composition according to claim 4 to 6 comprising bringing an immunogenic detoxified protein according to any one of claims 1 to 3 into association with a pharmaceutically acceptable carrier.
- 15 15. A method for the prevention or treatment of disease in a subject, comprising administering to said subject an immunologically effective dose of an immunogenic composition according to any one of claims 4 to 6.

1 / 12

FIG. 1

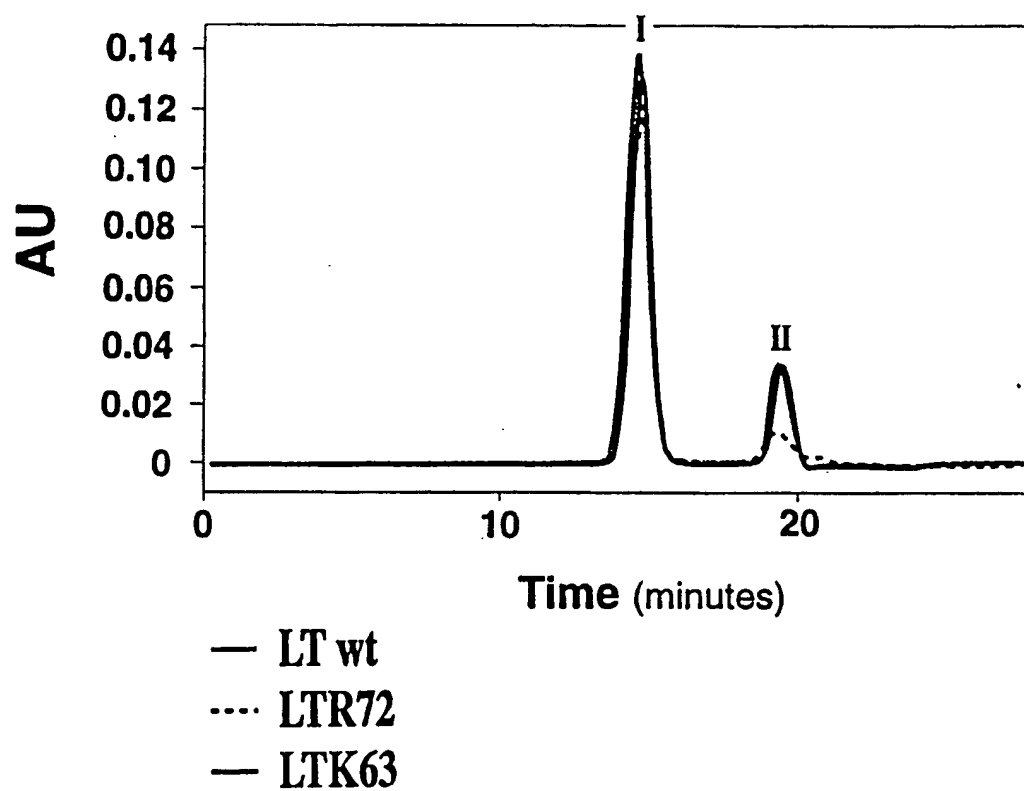


FIG. 2

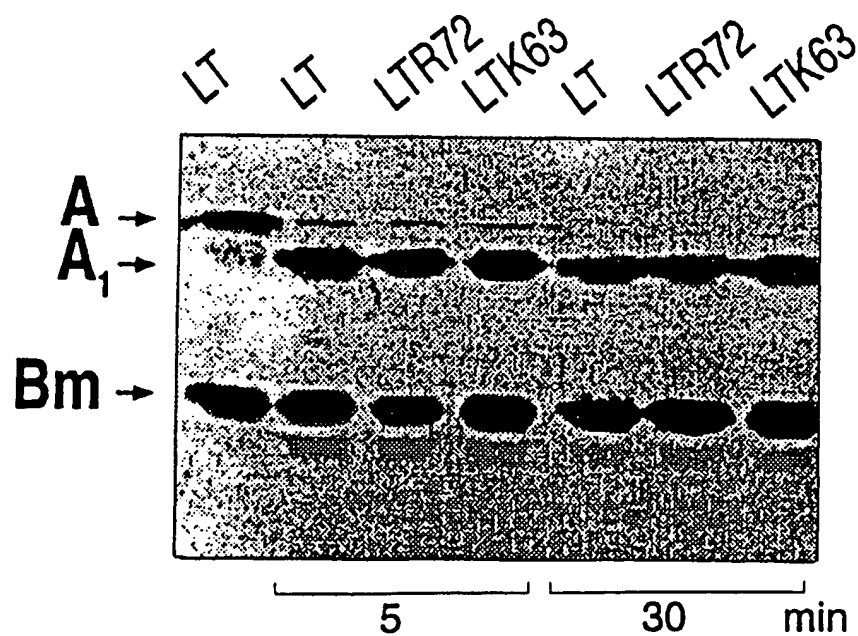


FIG. 4

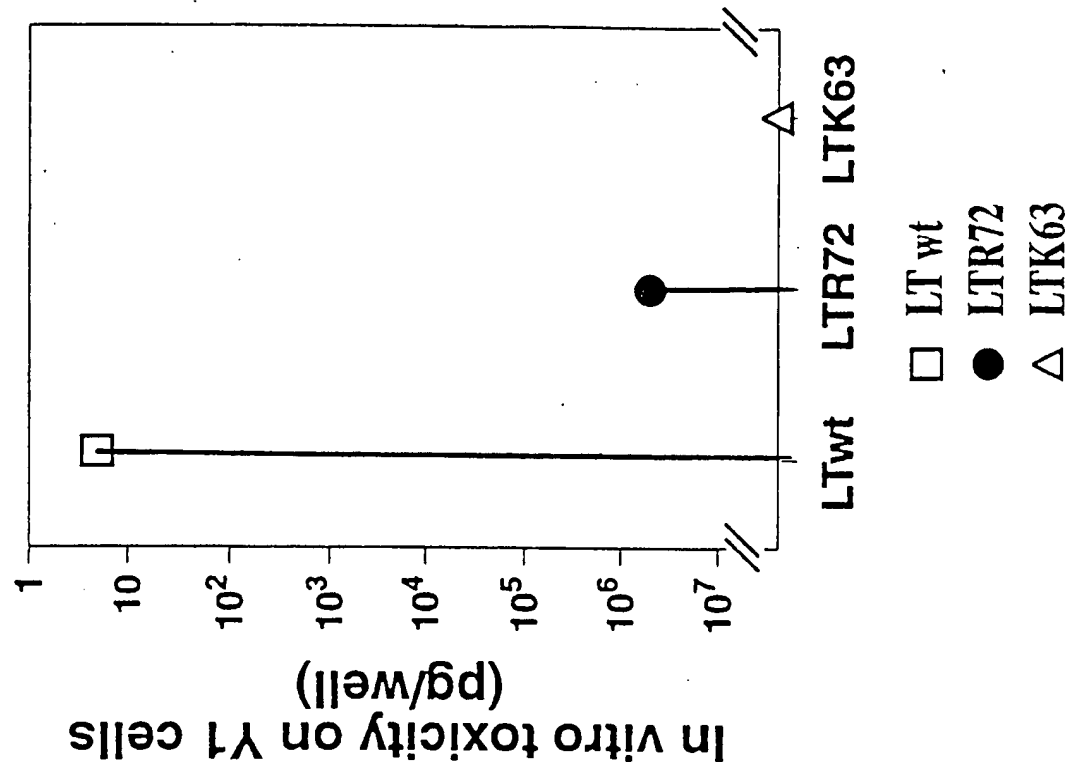
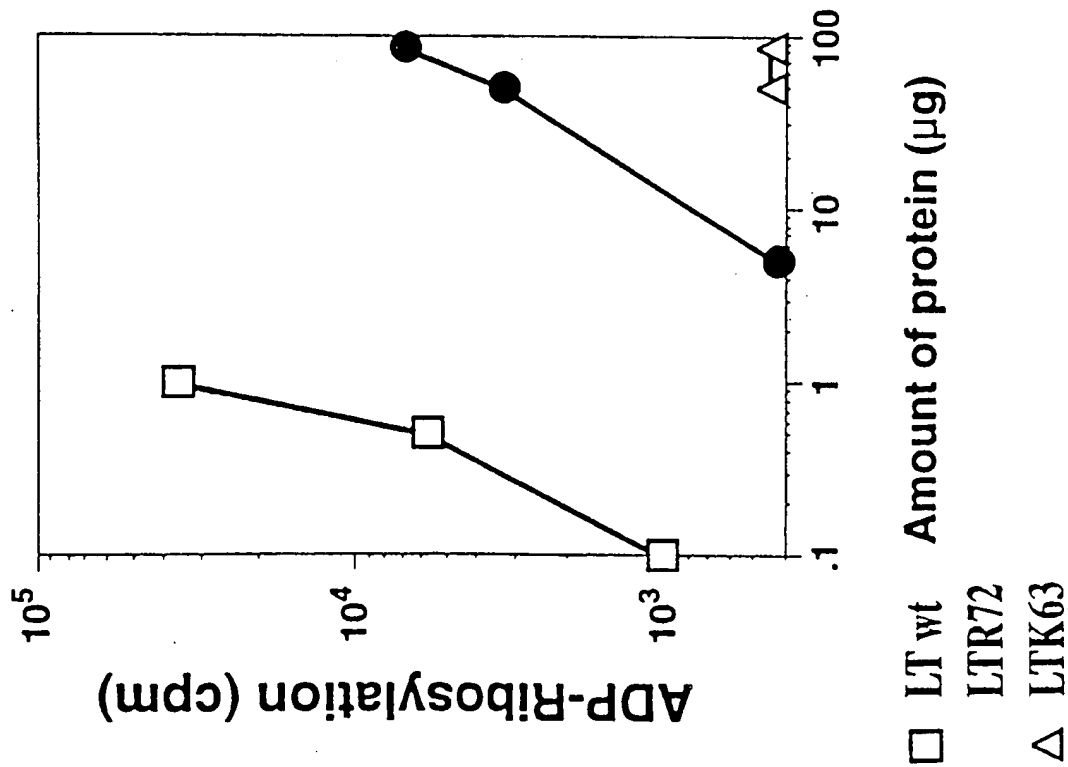


FIG. 3



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FIG. 5

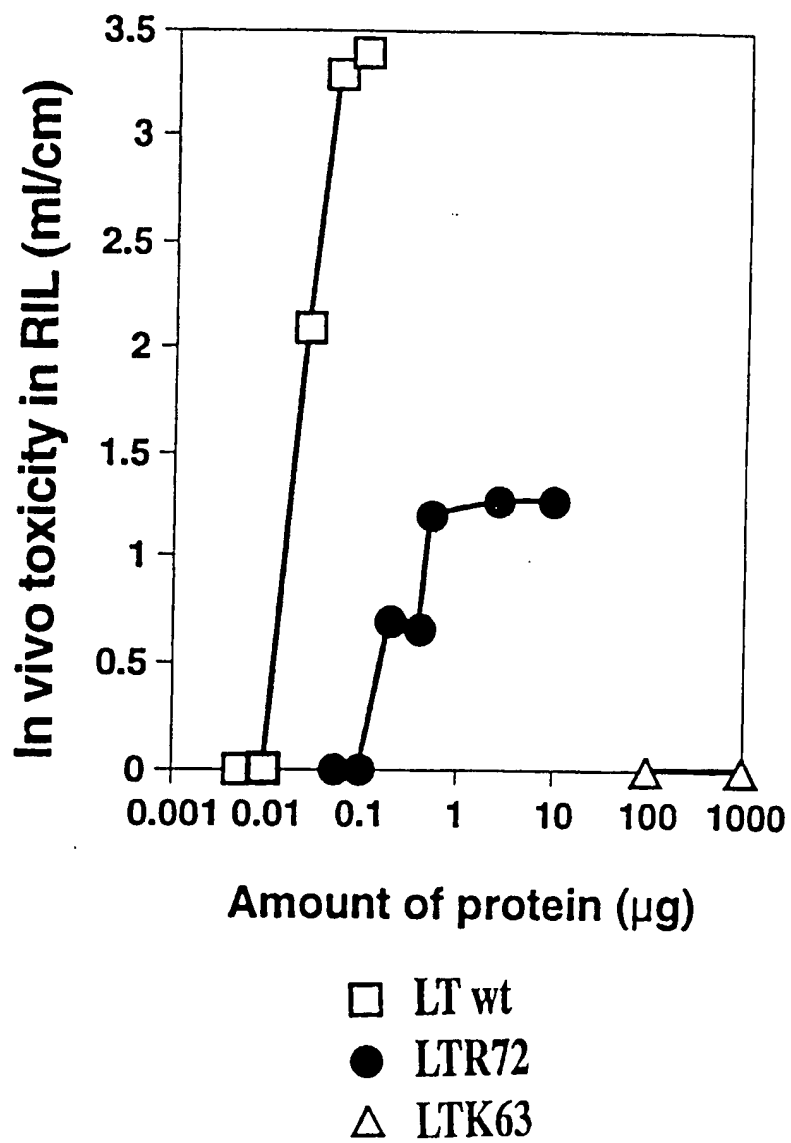


FIG. 6

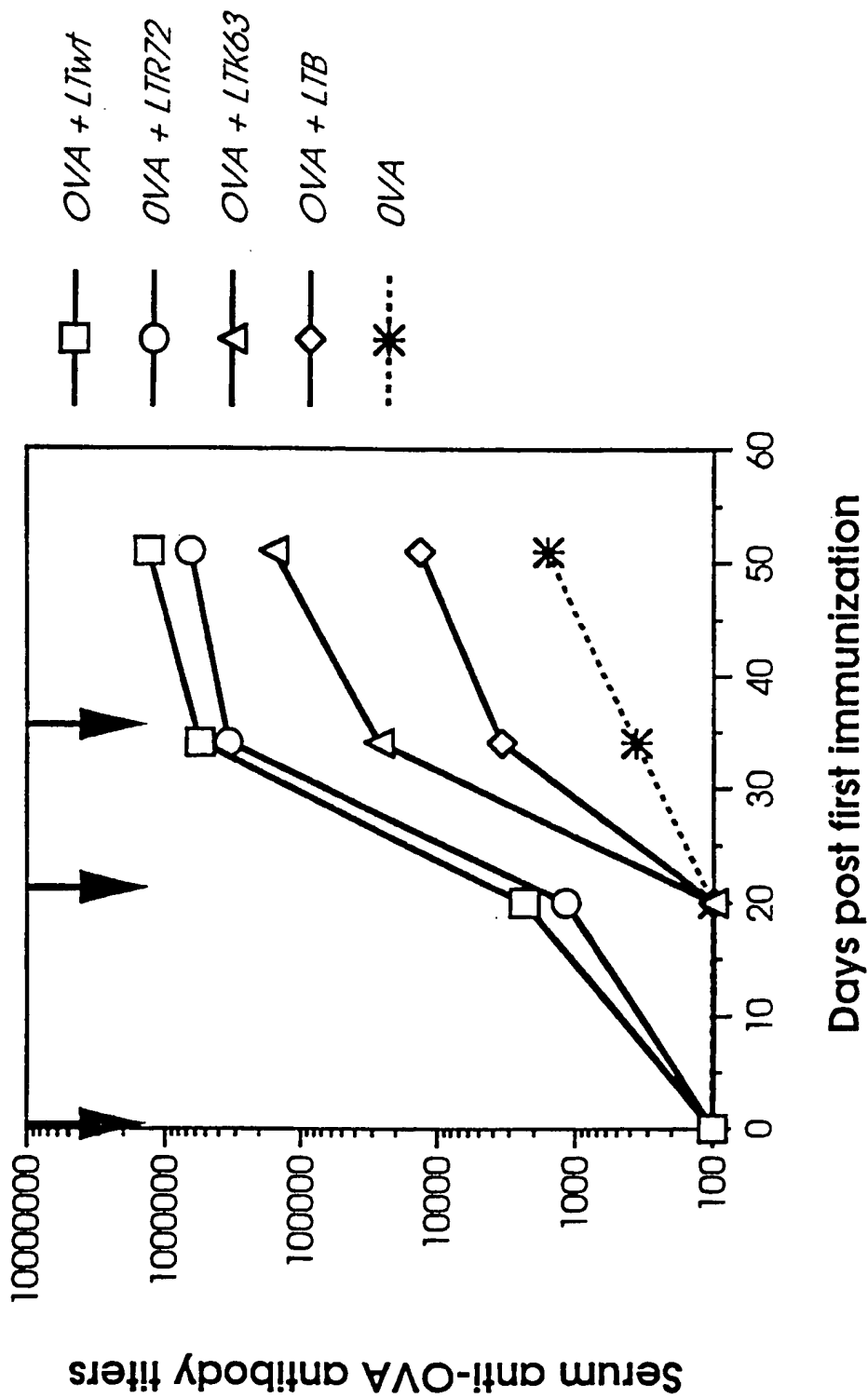
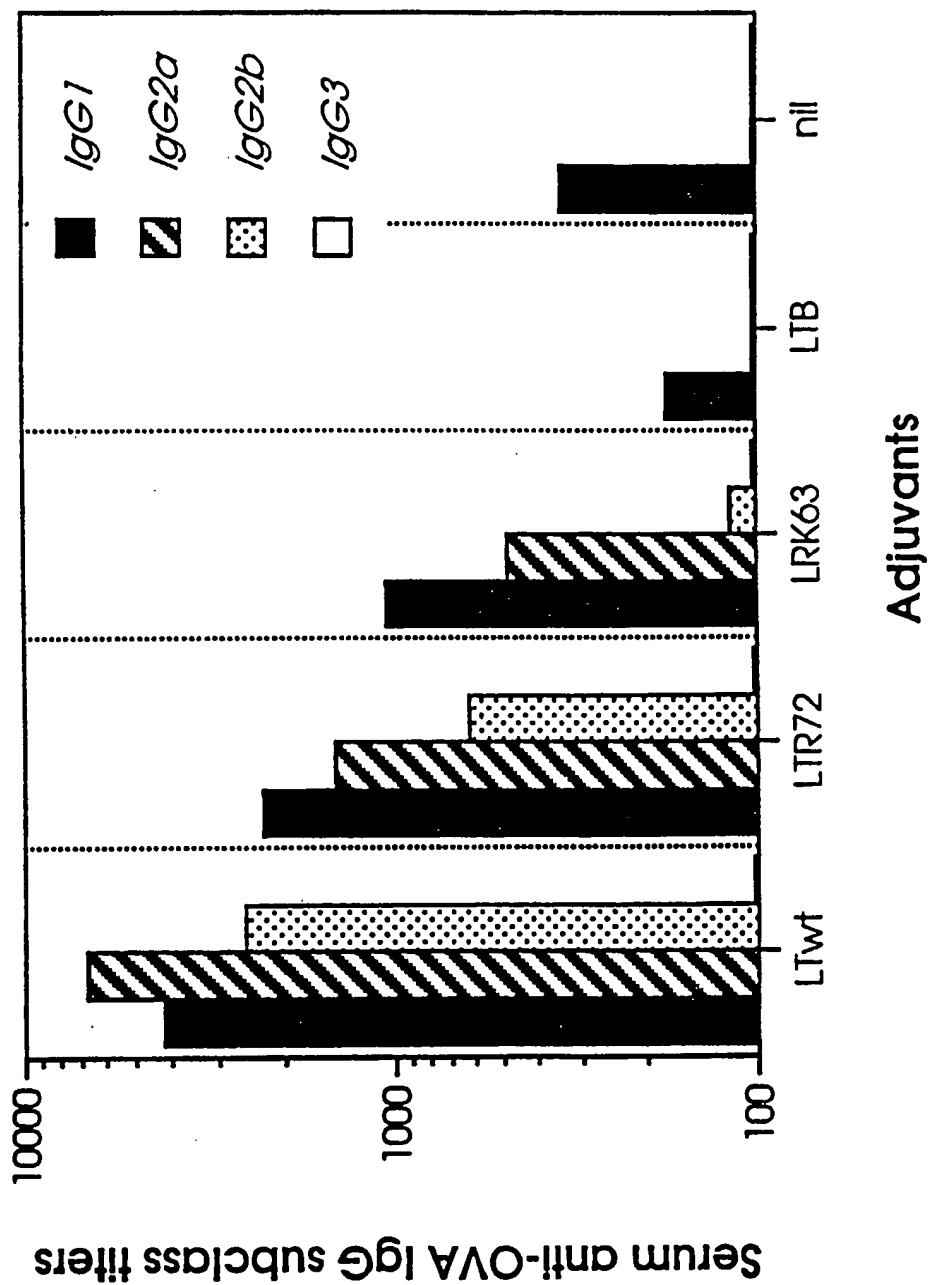
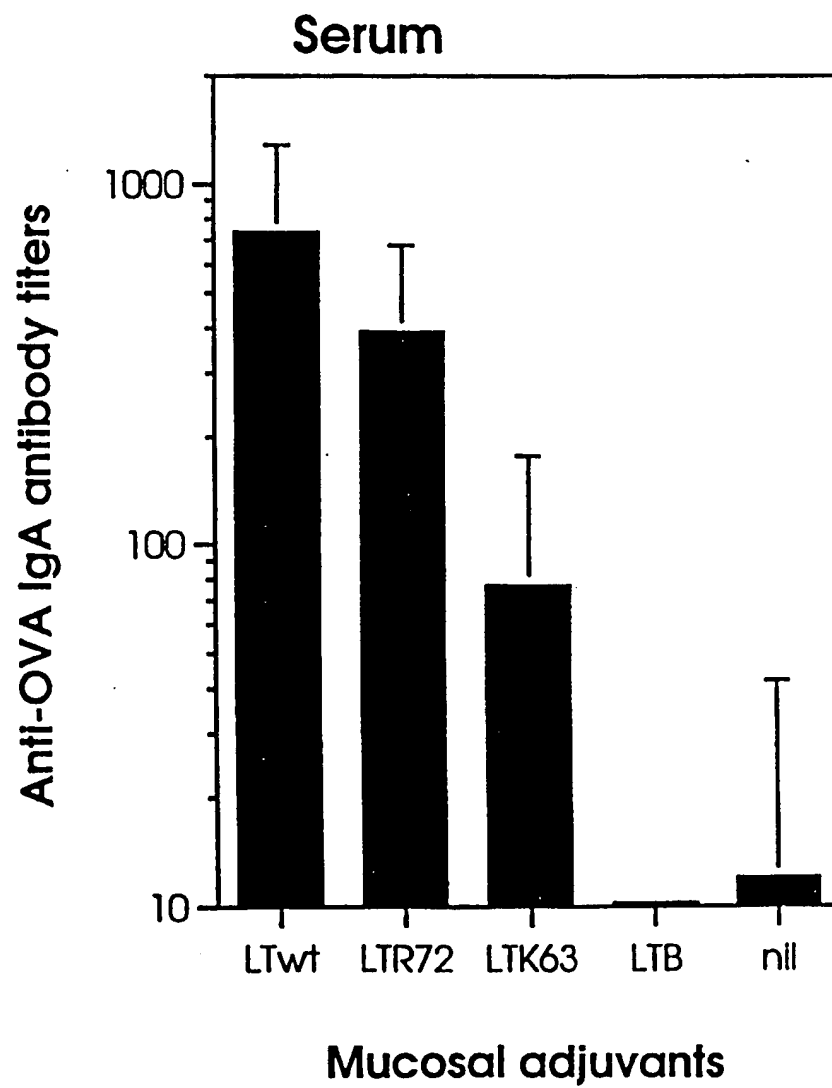


FIG. 7



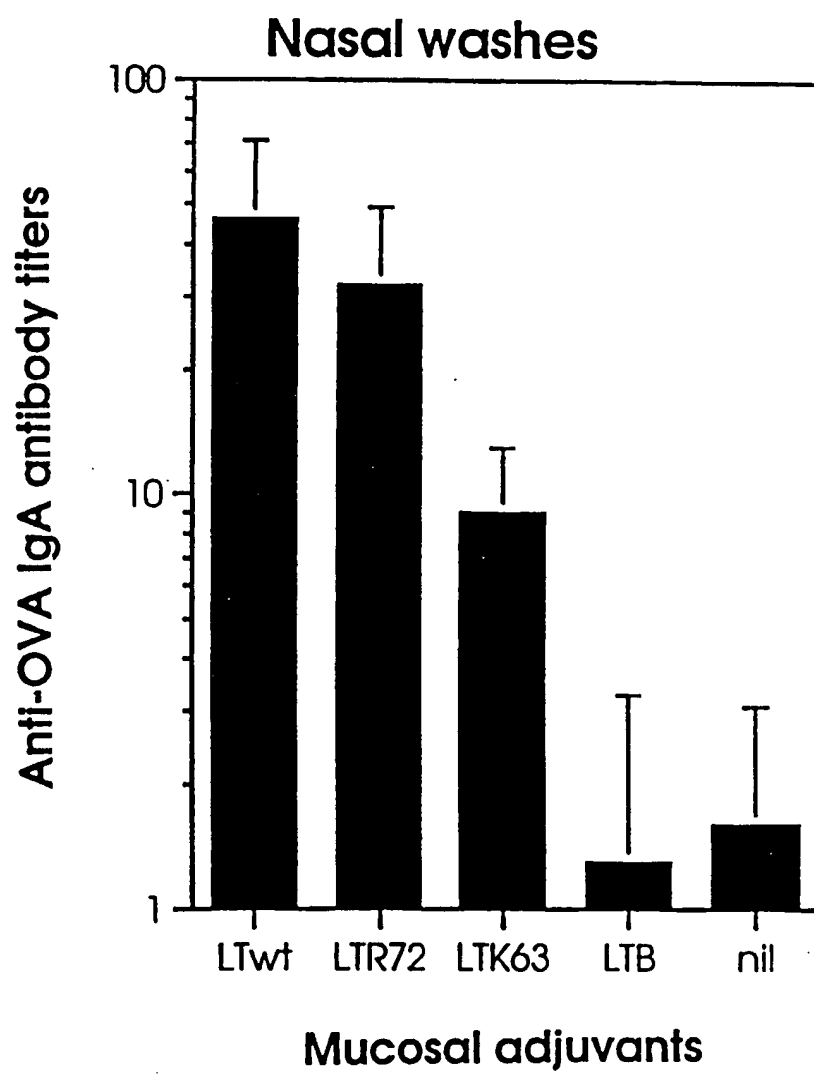
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FIG. 8(A)



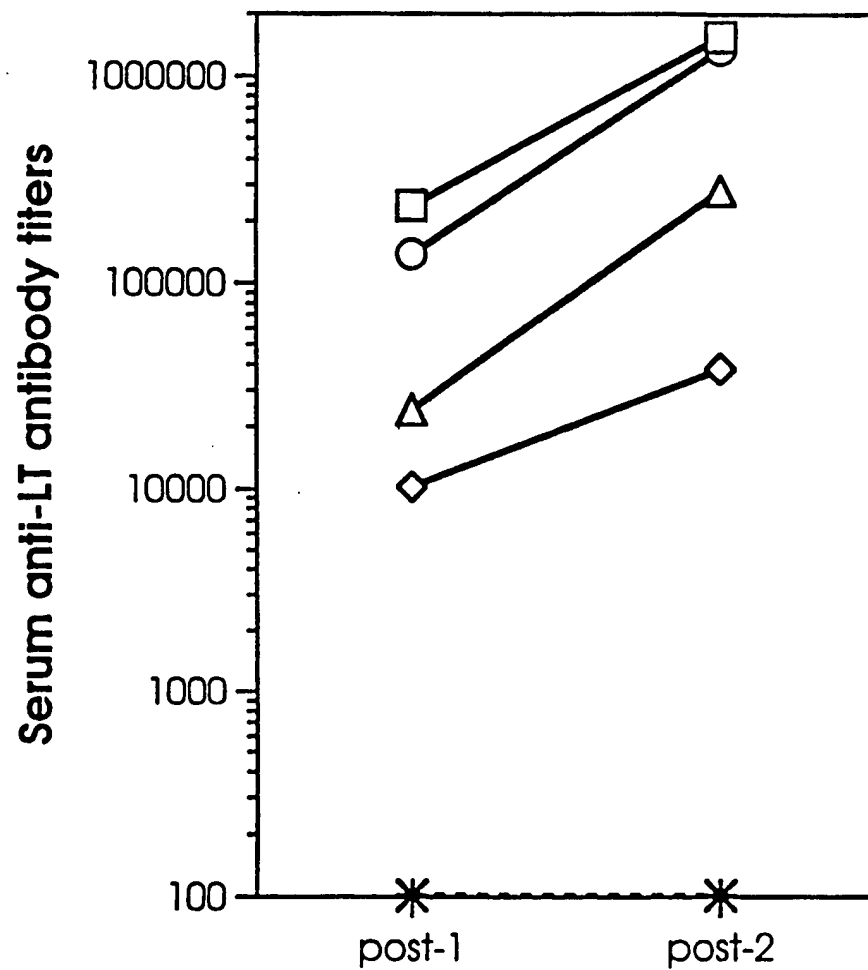
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FIG. 8(B)



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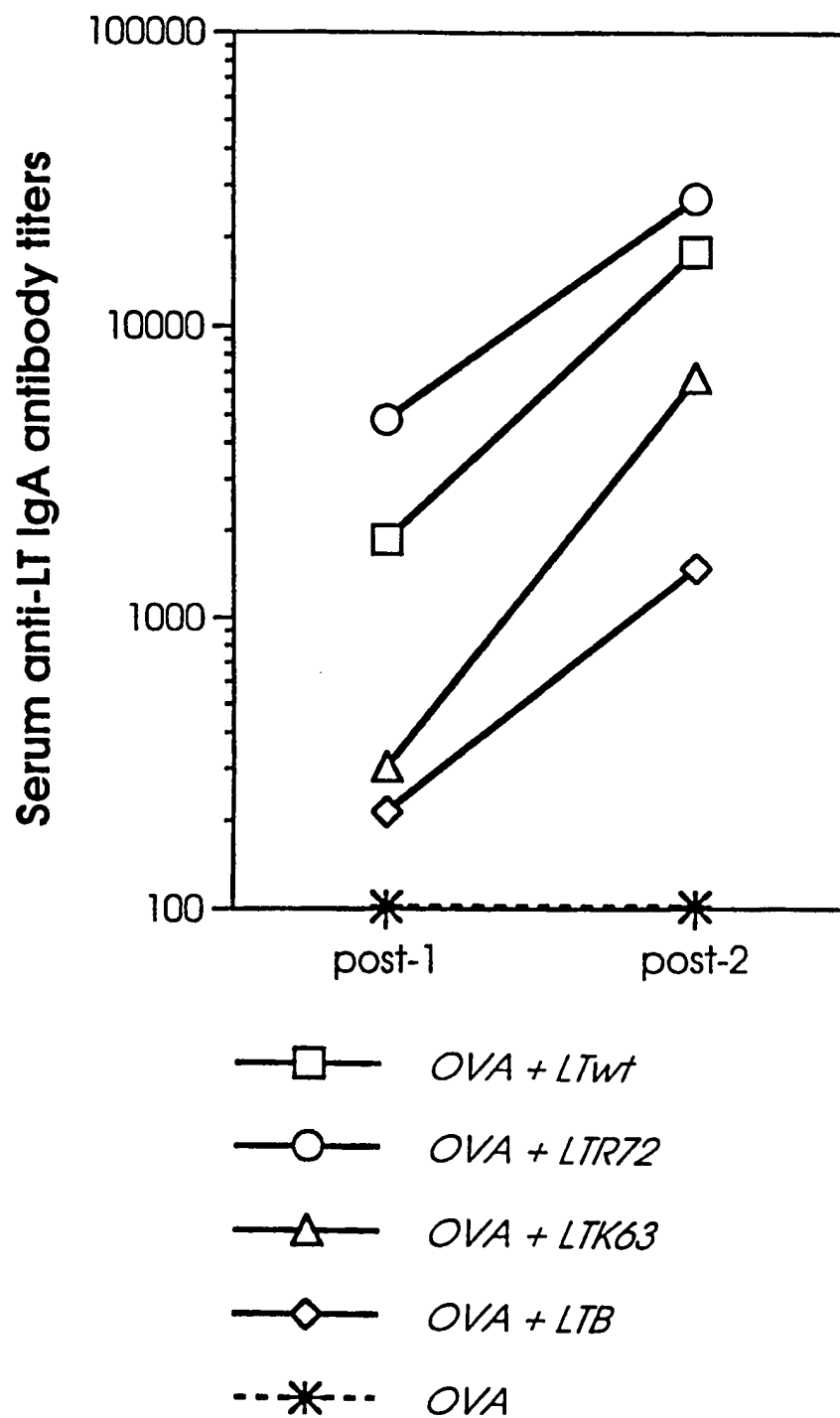
FIG. 9(A)



- OVA + LTwt
- OVA + LTR72
- △— OVA + LTK63
- ◇— OVA + LTb
- *--- OVA

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FIG. 9(B)



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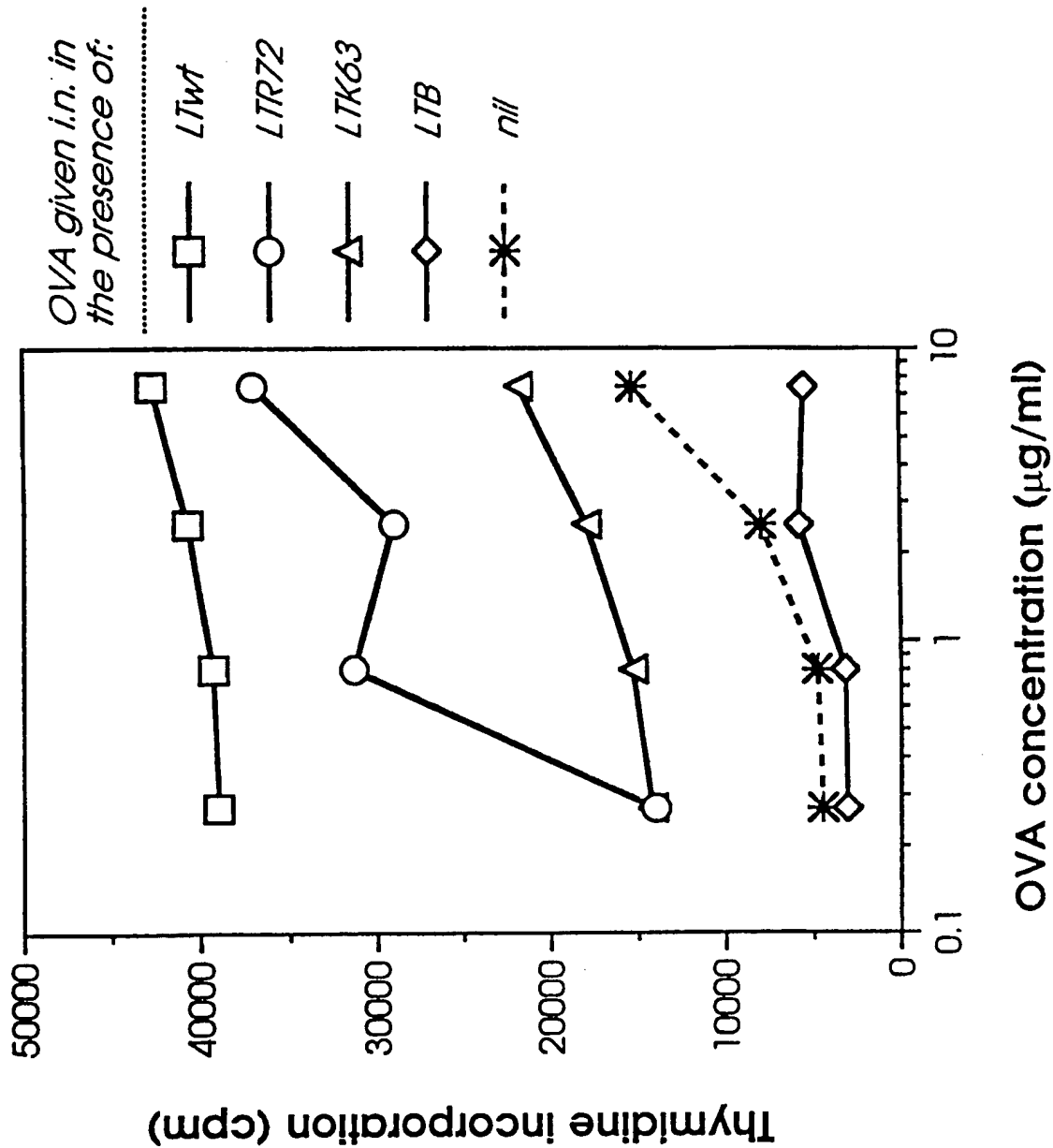
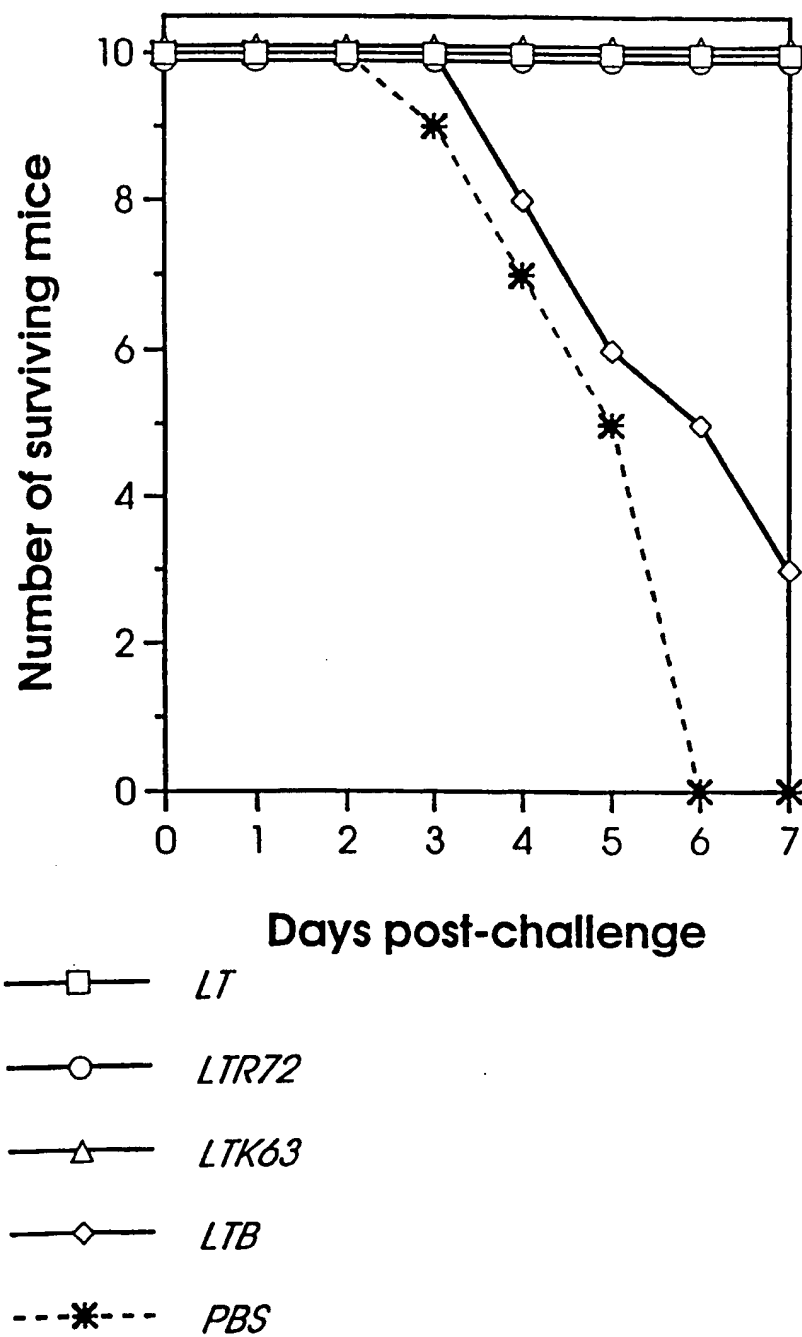


FIG. 10

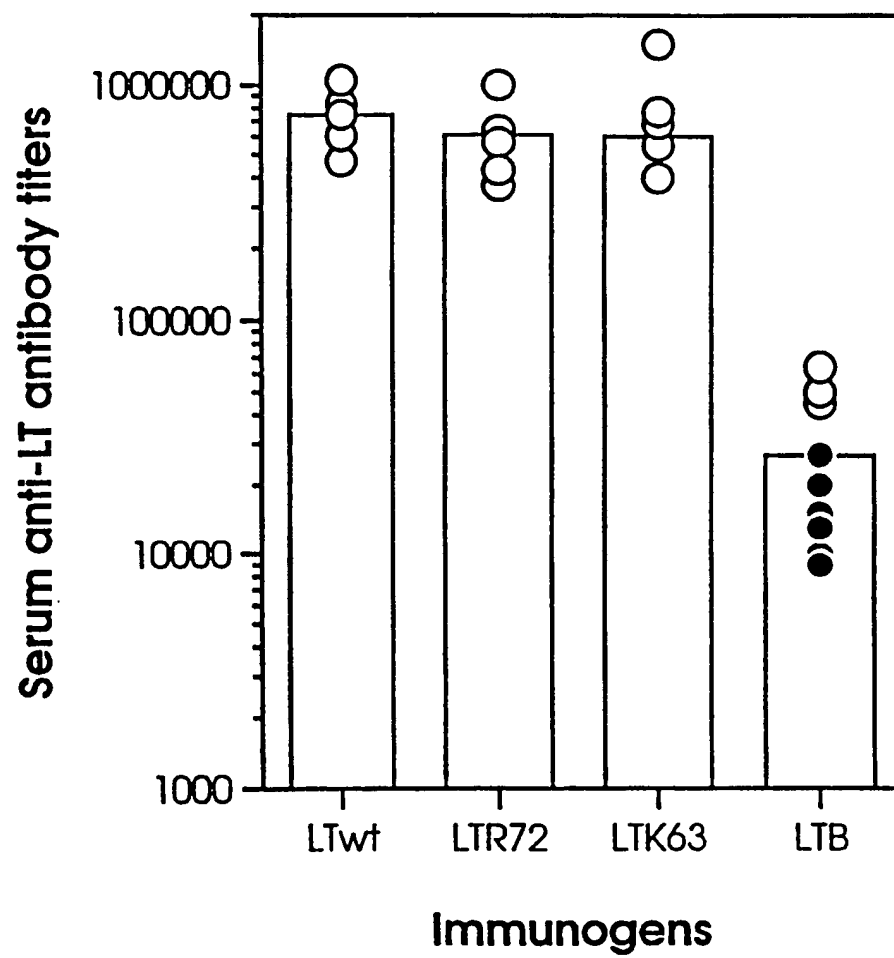
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FIG. 11(A)



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FIG. 11(B)



INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 97/01440

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/31 C07K14/245 A61K39/108 A61K39/39 C12N1/21
C12Q1/48 G01N33/50 A01K67/027 A61K49/00 //(C12N1/21,
C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K C12Q G01N A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 13202 A (SCLAVO BIOCINE SPA) 8 July 1993 cited in the application	1-5,8-15
Y	see page 46, line 51-52 see page 49 - page 50; claims see figure 2A	6,7
Y	DI TOMMASO A. ET AL.: "INDUCTION OF ANTIGEN-SPECIFIC ANTIBODIES IN VAGINAL-SECRECTIONS BY USING A NONTOXIC MUTANT OF HEAT-LABILE ENTEROTOXIN AS A MUCOSAL ADJUVANT" INFECTION AND IMMUNITY, vol. 64, no. 3, March 1996, pages 974-979, XP002054784 cited in the application see the whole document	6,7

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

Z document member of the same patent family

Date of the actual completion of the international search

12 February 1998

Date of mailing of the international search report

06.03.98

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 97/01440

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOUCE G. ET AL.: "MUTANTS OF THE Escherichia coli HEAT-LABILE TOXIN LACKING ADP-RIBOSYLTRANSFERASE ACTIVITY ACT AS NONTOXIC, MUCOSAL ADJUVANTS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 92, February 1995, pages 1644-1648, XP002054785 cited in the application see abstract see page 1646; figure 2 ---	6,7
Y	WO 95 17211 A (BIOCINE SPA ;RAPPUOLI RINO (IT)) 29 June 1995 cited in the application see page 5, line 9 - page 6, line 28 see page 8, line 34 - page 9, line 19 see page 15, line 19-37 ---	6,7
Y	WO 96 06627 A (UNIV TULANE) 7 March 1996 see page 13, line 33 - page 15, line 1 see page 30, line 7 - page 32, line 34 ---	6,7
X	PIZZA M. ET AL.: "PROBING THE STRUCTURE-ACTIVITY RELATIONSHIP OF Escherichia coli LT-A BY SITE-DIRECTED MUTAGENESIS" MOLECULAR MICROBIOLOGY, vol. 14, no. 1, 1994, pages 51-60, XP002054658 see abstract see page 57, left-hand column, paragraph 5 - right-hand column, paragraph 1 ---	1-3,9-13
A	VAN DEN AKKER F. ET AL.: "THE ARG7LYS MUTANT OF HEAT-LABILE ENTEROTOXIN EXHIBITS GREAT FLEXIBILITY OF ACTIVE SITE LOOP 47-56 OF THE A SUBUNIT" BIOCHEMISTRY, vol. 34, 1995, pages 10996-11004, XP002054659 see page 11002, right-hand column, paragraph 1 ---	1
A	EP 0 145 486 A (GLAXO GROUP LTD) 19 June 1985 see page 16, line 17-19 see page 18, line 3-6 see page 18, line 33-37 see page 21, line 23-24; figure 1 -----	1,6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 97/01440

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/IB 97/01440

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claims 8 and 15 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB 97/01440

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9313202 A	08-07-93	IT 1253009 B	10-07-95
		AU 3347693 A	28-07-93
		CA 2127091 A	08-07-93
		EP 0620850 A	26-10-94
		JP 7506240 T	13-07-95
		MX 9207685 A	31-05-94

WO 9517211 A	29-06-95	AU 1278595 A	10-07-95
		CA 2179771 A	29-06-95
		EP 0732937 A	25-09-96

WO 9606627 A	07-03-96	AU 3233795 A	22-03-96
		CA 2198586 A	07-03-96
		CZ 9700562 A	15-10-97
		EP 0777490 A	11-06-97
		FI 970799 A	24-04-97
		NO 970993 A	25-04-97
		PL 318930 A	21-07-97
		ZA 9506412 A	11-03-96

EP 0145486 A	19-06-85	AU 3644784 A	04-07-85
		DK 592284 A	13-06-85
		JP 61005097 A	10-01-86
